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Scientific contributions of Pierre Gadal and his lab—A tribute to Pierre Gadal (1938–2019)

Running head: Scientific contributions of Pierre Gadal and his lab

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 The corrections made in this section will be reviewed and approved by a journal production editor.

Q3 Q4

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Q5

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Q6

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Abstract

This chapter is dedicated to the memory of Pierre Gadal a former Series Editor for *Advances in Botanical Research*. Its aim is to describe the research that has been done in his laboratory covering essentially the period 1970–2000 and provides first-hand testimony about how it has been done. Besides the scientific output concerning CO₂ and nitrogen assimilation and their regulation described in this chapter Pierre Gadal has been very influential in plant science in France through his interactions with the University system but also with CNRS and INRA communities. Through the organization of the doctorate school in University of

Paris-Sud he has helped train hundreds of students, and more specifically concerning his own research group, more than 10 of his former students have become CNRS researchers, many with high positions and responsibilities, a similar number INRA researchers and Professors and Assistant Professors in the University system. Fourteen different colleagues have contributed to this chapter, each with his own perception, heart and guts, a further testimony to the exceptional human and scientific qualities of Pierre.

07 1 Pierre Gadal's career

Pierre Gadal was born in a modest family of farmers in Brittany in France, and he initially spoke only the Gaelic dialect. It was only in primary school that he got trained in French (amazingly enough his French grammar and spelling was perfect despite the slow take-off). After high school he went to University probably in Rennes and then started a PhD on galls and the metabolism of tannins in plants in the laboratory of Pr A. Brunel in Toulouse. Pierre Gadal has then become assistant professor in Université Paul Sabatier in Toulouse. He later became a full professor at Université de Nancy I in Lorraine (1973–1981) and finally at Université de Paris-Sud Orsay until the end of his career in 2003. He has headed a CNRS-associated lab (Physiologie Végétale Moléculaire) in Nancy and later in Orsay to finally become the initiator and the first director of Institut de Biotechnologie des Plantes (CNRS-Université Paris-Sud), an institute which played a very important crucial role for the structuration of plant biology around Paris. Pierre Gadal has had a large number of numerous pedagogic and administrative responsibilities, in particular he has supervised the training of PhD students at Paris-Sud during many years. Pierre Gadal has been very influential in the domain of plant physiology in France and was awarded a state prize from the French Academy of Sciences in 1998 as a crowning of his scientific career. In 2000 he was elected as a Corresponding Member of the American Society of Plant Biologists. When he officially retired in 2003, a special issue of the *Journal Plant Physiology and Biochemistry* has been dedicated to him. His scientific contributions have gained international recognition with more than 200 articles published in peer-reviewed journals. More specifically his major scientific contributions were linked to the elucidation of the regulation of CO₂ fixation in plants through the study of PEP carboxylase and NADP-malate dehydrogenase, but also to the study of the pathways allowing nitrogen assimilation in plants (mostly glutamine synthetase and GOGAT). His latest interests concerned the carbon-nitrogen relations in plants involving isocitrate dehydrogenase. Pierre Gadal and his group have been leaders in plant biochemistry, introducing in the late 80s the use of genetic engineering and structural biology. After his retirement, Pierre Gadal has been a series editor for *Advances in Botanical Research*, a book series by Elsevier, supervising volumes 63–86. As an emeritus he was also a very highly appreciated advisor for students belonging to the doctoral school of Orsay Plant Sciences, helping them to find challenging post-doctoral stays. He died in October 2019 after fighting a difficult disease. Reactions to his departure have been unanimous: this was a major actor of plant biology who has disappeared. All the students and colleagues who had the chance to work with him have been impressed by his charisma and enthusiasm. He was always stimulating us to go forward and in the same time he paid extreme attention to his colleagues helping them as much as he could, irrespective of their social positions. He will be thoroughly missed by all of us. In the following section of this chapter several of the members of the Gadal lab pay tribute to Pierre by recalling how science was done there and the major achievements that were produced.

2 Mechanisms and regulation of CO₂ fixation in C₃ and C₄ plants

2.1 PEP carboxylase

2.1.1 Jean Vidal's recollections

This short text summarizes the scientific contributions from P. Gadal's laboratory at the University of Nancy and then at the University of Paris-Sud in Orsay, by the people cited in the below acknowledgements. It is intended to illustrate, via some selected memories, a long period of interaction with this outstanding plant scientist and teacher. To avoid enumerating a long list of scientific details (already published elsewhere in International scientific journals) I have decided to briefly revisit, in an informal, elliptical style, some milestones of the corresponding periods. In this way, the people cited below and I express our deep gratitude to Professor Pierre Gadal (PG).

Toulouse, October 1973: Centre de Physiologie Végétale, Université Paul Sabatier (Director, G. Brunel); on the evening of the same day I had presented my thesis defense: “My application for a Professor position has been positively considered at Nancy University, would you be interested to move there as a CNRS researcher and to participate in the

installation and development of a new Plant Physiology laboratory?," PG asked the newly graduated PhD student that I was. I could not believe what I heard but I managed to answer "Yes of course!!!" with a stammering voice. This short dialog with PG was the starting point of my long (actually up to my retirement in 2010) professional collaboration with him. Until then, I had been living in a dilettante way, with no concrete objective for my career, although I remember when regarding the functioning of biological systems, I was constantly haunted by the fundamental, mechanistic, quasi existentialist question: How does it work? In what follows, and essentially thanks to PG, it will appear that my wishes were ultimately granted.

Before I develop my personal story further, I think it is indispensable to underline the seminal impulse of Professor G. Cavalié, director of my thesis work centered on the study of two generally coupled enzymes of primary plant metabolism, namely, phospho*enol*pyruvate carboxylase (PEPC) and NADP-dependent malate dehydrogenase (NADP-MDH). Later, he allowed this scientific project to be transferred to Nancy.

Nancy, October 1974: The fact that most of the laboratory had to be entirely set up raised the paradoxical feeling of concern (energy and time-consuming for, perhaps, little scientific yield and rewards) and unalterable enthusiasm when considering our contribution to this noble goal. Again, PG was leading us with his infectious, optimistic personality, conferring enormous doses of confidence for the future to the people around him and, thus, provoking a strong impetus; nothing and no one could have stopped the machine.

As expected, the formerly classical botany laboratory had neither the necessary equipment to do modern biochemistry nor space for controlled plant cultures. It was indeed a desert, but we never felt discouraged; on the contrary, this adverse situation turned out to be a very stimulating period since the first year was devoted to laying down the foundations of the new laboratory, setting up minimal working conditions, together with the two very first students of the nascent group (J. Maréchal and M-C. Rio) and, I must say, the precious help of colleagues in the Biology Department. Cultivating, centrifuging, measuring and more in neighboring laboratories was the rule; in fact it was just necessary to have a good ice machine and buckets to transport fragile materials!!! What an intense pleasure it was—the inauguration of a cold room for plant extraction and experimentations—the arrival of a newly purchased spectrophotometer. Our efforts converged toward the biochemical characterization of the two above-mentioned enzymes and the regulatory mechanisms impacting on their activity. I remember that this period was exceptionally stimulating and enriched by permanent, informal discussions with Pierre as our scientific mentor. At the same time, he was organizing laboratory activities, including boring administrative tasks, internal meetings and useful collaborations nearby and around the world, while also teaching plant physiology. His charisma made his course so attractive that it was quickly attended by an evergrowing number of young students (see J-P. Jacquot, this issue). This had the advantage of getting more students interested in plant sciences and, more importantly to us, in research options developed by the group. Already during the second year of the laboratory's existence, this option took shape with the addition of three more students: C. Guiz, J-P. Jacquot, and N. Galvaing, all surmised as promising elements by Pierre (H. Membre, F. Martin A. Suzuki, and B. Hirel would follow shortly later). In the ensuing years, together with J-P. Jacquot, exciting research on NADP-MDH was developed, with the identification of a redox-activating factor, later to be named thioredoxin. This was largely amplified by J-P. by interactions and stays abroad, most notably in the group of Professor Bob Buchanan (a world leader in plant enzyme redox regulation) at UC-Berkeley in the USA. During the next period at Orsay (see below), J-P. developed a research group that rapidly expanded to produce a wealth of new and exciting results so that they quickly reached the pinnacle of the thioredoxin field (cf J-P. contribution, this issue).

As for myself, I concentrated on PEPC in the context of C₄ photosynthesis and light, and the fascinating application of immunological techniques to plant proteins that led to a technical breakthrough that brought considerable progress. Up until then, to clarify functional parameters of proteins and to allow us to make hypotheses about their metabolic impact, we purified enzymes by a series of very time-consuming chromatography techniques. High purity (but never perfect purity) was achieved in this way, but this was not the guarantee that alterations did not occur and generate super artifacts and thus lead to invalid conclusions. PG had been harassing me with the question of the production of PEPC antibodies. Consider it done, I just had to contact our next-door neighbor, Professor G. Godbillon at the Animal Physiology laboratory, and thanks to him I learned how to prepare the precious matter and how to use it (a savoir-faire that was perfected later by a stay in J. Daussant's laboratory at POVAR, CNRS Meudon). It has to be said that this was a particularly enjoyable and productive period during which, together with our recently recruited, excellent technician J. Gerard, we set up a fast and efficient one-step purification procedure (immunochromatography with water elution) of the photosynthesis related C₄ form of PEPC that was collected quasi free of contamination. This also opened up the

rewarding period of in situ immunolocalization studies. The actors brought together by Pierre were newly recruited C. Perrot (later Catherine Perrot-Rechenmann), and G. and A. Bulet at “Hôpital Universitaire de Brabois,” who had a very good knowledge of such approaches on human or animal samples, both on their theoretical and technical aspects. The question at the time was: could immunolocalization be applied to plant systems? The answer was a resounding “Yes!,” and by using this immunological tool, Catherine clarified another highly debated question of the time, namely the tissue, cell and subcellular localization of C₄-PEPC. She produced the first, very impressive, truly artistic, but nevertheless precise images showing the cytosolic localization of the enzyme in Sorghum leaf mesophyll cells. This result was of paramount importance since it allowed to place this metabolic component at its correct position in the cell, and consequently inferred the organization and functioning of the C₄ photosynthesis-linked metabolic network in the entire leaf.

We also established that the light-induced accumulation of the very abundant C₄-PEPC in the mesophyll cells was due to neosynthesis and, as a corollary (what was another important issue at the time was whether the chromatographic-based enzyme diversity was true or due to experimental artifacts) the occurrence of PEPC isoforms thereby likely implicating a corresponding multigene family. Meanwhile, a long-term interest of PG had been achieved via the installation of a new research activity, devoted to the study of plant nitrogen metabolism, alongside that of carbon. He had been eagerly waiting for such a possibility, as he thought that a comprehensive, integrated approach of primary plant metabolism necessarily involved both aspects (refer to nitrogen contributors, this issue). The CNRS soon recognized the merits of PG's vision by attributing his team a CNRS-associated group label, which was a deserved and important distinction for him and his lab.

Orsay, October 1981: But everything has an end. Pierre was offered a position at the faculty of Sciences (Université Paris-Sud, Orsay) as the replacement of Professor Moysé (Laboratory of “Physiologie Végétale”). We all followed him there, even though it was quite difficult to leave behind what had been painstakingly built-up at Nancy II; a nice scientific environment, lovely people (both inside—M. Jacquemin, B. Maudinas, G. Chemardin, J.F. Pierre, H. Courbet, R. Beauval and the administrative staff—and outside—groups within the Biology department) and the gorgeous Lorraine region. The foreigner that I was, strangely and unexpectedly felt uprooted.

History repeats itself! Curiously enough for such a well-respected University (dating from the origins of the faculty in 1956), we first had to renovate the old laboratory in Building 430. Even the electricity did not meet the safety norms. Again, PG was particularly active and efficient in striving to insert his group into this new, particularly rich, biochemical and physicochemical environment of a much broader dimension. When functionality was finally attained, the time had come to enter a new scientific era of molecular biology and genetics. For lack of space, I must make a very long story short. This era was initiated by M. Thomas and C. Créatin, pursued and nicely developed later by Claude's student, L. Lepiniec. Martine was concentrating her interests on the molecular mechanisms controlling light-activated synthesis of the C₄-PEPC protein. Briefly, using sophisticated techniques including monoclonal antibodies (in collaboration with J.M. Bidart, IGR, Villejuif) and in vitro transcription analyses (collaboration with Dr. E. Mosinger, University of BERN), she elegantly showed that leaf greening was accompanied by an increase in the production of C₄-PEPC encoding mRNA, and that this was phytochrome-controlled.

On his side, Claude had been learning abroad (Max Planck Institute in Munich with Professor Hahlbrock) how to develop and use a variety of new techniques that any research group had to possess to face the challenges of the moment and notably, DNA cloning, genomic libraries, production of recombinant proteins and plant transformation that were successfully applied to the PEPC theme. Claude conducted his research with a technician, Eliane Keryer, and a PhD student, Loïc Lepiniec. Three genes of the sorghum PEPC family were isolated, sequenced and their expression clarified. Their nucleotide sequence allowed revealing their deduced amino acid sequences and specific domains, like the N-terminus containing a conserved serine residue which was receiving special attention at the time in the other part of the PEPC group. Biolistic experiments and reporter gene expression allowed the tissue expression and the specificity of PEPC gene promoters to be analyzed. Several years later, in relation to this, a Polish PhD student, S. Rydz (Warsaw University, Professor A. Richter) detected protein factors with an affinity for the C₄ gene promoter that were possibly regulated by protein phosphorylation.

However, in August 1993 a very dramatic and sad event occurred when Claude passed away. It was a great loss, both scientific and human, that struck everybody who knew him.

Then in 1994 came the moment to move from the banks of the Yvette river to the heights of the Saclay plateau where stood a magnificent new building called the “Institut de Biotechnologie des Plantes” or IBP for short. This Institute was in all aspects, from the idea of its creation to the raising of its walls, a PG project with the later collaboration of Professor F. Quétier. At the time, the vision of PG was met with some indigenous resistance and some strong opposition from the CNRS department of Life sciences, but PG was not deterred in his demonstration of a vision for the future of Plant Sciences at Orsay and his strong character. This big building with an elegant glass greenhouse housed around 150 people including 100 researchers/teachers working in three domains of plant sciences; development, plant-microbe interactions and metabolism. In fact, it was so attractive, especially when compared to the rest of the campus, that the faculty used to send, for years, foreign delegations to visit the “fleuron” of the campus buildings. PG became the director of the IBP, which was awarded an UMR label by CNRS.

After the closing of the “Institut de Physiologie Végétale” (CNRS Gif/Yvette) at around the end of the 1990s, several O. Queiroz group members joined the PEPC group at the IBP. J. Brulfert (with PhD students Tahar Taybi and Nadia Bakrim) brought her excellent knowledge and problematics of CAM PEPC, Kim Tran (and Van Le Bui) developed useful and original techniques for plant transformation, while J-N. Pierre mastered a very precious technique offering the potential for in-cell integrated approaches. We had shown that sorghum C₄-PEPC became phosphorylated in illuminated sorghum leaves by an elusive PEPC kinase (PEPCK). The identification of the kinase was an important challenge. Making use of recombinant protein technologies, V. Pacquit, N. and N. Bakrim, M. Nhiri and C. Echevarria (University of Sevilla) were studying C₄-PEPC and its phosphorylation in vitro, showing that calcium-dependent kinases could be candidates for the role, at variance with Professor R. Chollet's group (Lincoln, Nebraska, USA) which was proposing a calcium-independent kinase. The long debate came to an end when Professor H. Nimmo (Glasgow, UK), and quasi simultaneously R. Chollet, and J. Cushman (Reno, Nevada, USA) achieved the cloning of the authentic PEPC kinase in 1999 and 2000, respectively. Indeed, this kinase did not require calcium to phosphorylate the target serine of the PEPC N-terminus. In addition, it was amazingly small for a Ser/thr-kinase (only 32 kDa, untiringly neo synthesized every day at the beginning of the light phase, nevertheless of incredibly low abundance, and dedicated to PEPC. However, signaling systems are very intricate and particularly complex and biological knowledge is never complete so it can be hypothesized that our calcium-kinase was perhaps useful in some, as yet unknown, specific physiological condition. Valerie also produced the first C₄-PEPC crystals (collaboration with C. Cambillau of the CRMC2, Marseille) for X-ray diffraction analyses, but K. Izui (Kyoto University, Japan) was the first to publish the first, eagerly awaited, maize C₄-PEPC protein structure. It was with an extreme deep emotion (the dream had come true) that I, along with others, discovered the details (folding, N and C-termini, phosphorylation and active site domains, homotetramer) of the wonderful PEPC structure. It was certainly a nice protein structure, actually a dimer of dimers, but above all, it was an extremely useful tool to inspire the design of future experimental approaches. It was also the time to begin what was to become a long lasting and particularly rewarding collaboration with R. Chollet (Lincoln, East Campus then G. W. Beadle Center), an eminent US Plant biochemist who was (and still is) a reference in C₃/C₄ photosynthetic enzymes and mechanisms, and C. Echevarria, who had just obtained a professor position and taken over as head of the laboratory of “Fisiologia vegetal,” Sevilla). With them, a very active research network was built-up that allowed various short sabbatical visits and a dense exchange of PhD students and postdocs, all of them carrying out their thesis work on PEPC and/or PEPCK and thereby leading to an important number of publications.

The technique developed by J-N. Pierre opened up an exciting period dominated by cell biology to notably study how the light signal controls the synthesis of PEPC kinase in the cell. Again, it is difficult to try to describe the intense pleasure we felt at deciphering some key components of the transduction machinery. By using mesophyll cell protoplasts and both pharmacological and confocal microscopy approaches (S. Brown CNRS, Gif/Yvette), J-N. Pierre, N. Giglioli-Guivarc'h, and later S. Coursol and J. Grisvard, identified one by one a good part of the cascade components controlling PEPC kinase synthesis in the physiological context of the entire C₄ leaf. It was amazing to see how they were easily deducible from our knowledge of the spatial organization of C₄-photosynthesis in terms of both tissue concentric organization and metabolite exchange. Obviously much remained to be done but a paradigmatic, integrated scheme was now drawn to stimulate further investigations.

PG retired in 2003 and a tribute was given to him by way of a Special Issue of *Plant Physiology and Biochemistry* in June–July 2003 (see reference list). He then spent several years giving a hand to people (both teachers and PhD students) of the doctoral school and performing editorial work for *Advances in Botanical Research*. It was still possible to meet him to discuss our most recent research. The rapid progress of plant genetics, genomics and molecular genetic

techniques had made it necessary to constantly update the level and variety of investigations. Now, manipulating plants for biotechnological applications had become routine. It also provided fundamental research with extremely efficient and sophisticated tools to examine biological mechanisms with the holistic philosophy of Pierre, at the most integrated level possible. For instance, switching to the model C₃ plant *Arabidopsis thaliana* allowed the isolation of PEPC KO mutants from seed libraries in order to clarify the physiological role of leaf C₃-PEPC phosphorylation in situ. A. Gousset-Dupont made a good contribution to this topic, showing that this post-translational modification (PTM) was particularly needed to resist salt stress conditions. This approach on a C₃ plant was completed by B. Leboutteiller on PEPC mutants and overexpressors of the four genes encoding PEPC in *Arabidopsis* leaf and seeds. In collaboration with a private company, Biogemma (P. Perez, D. Gerentes), M. Jeanneau produced transgenic maize (over)expressing sorghum C₄-PEPC in all organs. Interestingly, these plants were significantly more resistant to drought when grown in a greenhouse. Unfortunately, when grown in the field, they were continuously cut-down by people opposed to transgenic plants or GMOs, and so the company (and us too) finally abandoned these experiments! At IBP, P. Meimoun had the honor of being the PhD student involved in bringing to an end the long story of PEPC. Before closing finally the doors of the PEPC laboratory at IBP, this “last of the Mohicans,” with the excellent assistance of F. Ambard-Bretteville, added an interesting contribution to C₃ PEPC phosphorylation via a generic study of the *Arabidopsis* phosphoproteome.

Today, clearly biological questions regarding plant metabolism and its regulation have shifted to other targets. Forgotten is the time when PEPC alone was attracting dozens of researchers worldwide to meetings (such as the one we had organized at the IBP as a satellite of the Xth International Photosynthesis congress, Montpellier 1995). To my knowledge, PEPC is still actively investigated by the groups of B. Plaxton (Kingston, Canada), C. Echevarria (Sevilla) and J. Cushman (Reno). It is both satisfying and reassuring to see that many unexpected findings were being revealed, among which I will underline only the PTM of the enzyme by regulatory mono-ubiquitination and the phosphorylation of C₃-PEPC in seeds. Although a considerable amount of data has been gathered from reductionism-based studies, the precise role of these PTMs remains obscure and the potential of more global, integrated approaches may help improve our understanding. So much is yet to be discovered and original properties might be expected to emerge. The great lesson learnt from practicing research along these many years, is that the permanent quest for/and acquisition of relevant techniques, concepts and bioinformatics-based models that invade the field, requires weaving inevitable and pertinent collaborations. Lastly, on a more biotechnological point of view, I also think that certain enzymes, having a particularly high capacity for fixing CO₂ have the potential to contribute in some way or another to fixing the rapidly growing challenge of climate change.

Above, I used the term of “mentor” to describe the profound influence of Pierre Gadal upon a group of young researchers hungry for plant science. Indeed, Pierre had all of the characteristics defining this word: an experienced advisor, attentive and wise and who deserves one's entire confidence. Although involved in teaching duties, laboratory organization and national and international responsibilities, PG constantly paid attention to the work performed in his laboratory. He was a continual driving force, always available for discussions, always introducing pertinent new ideas, concepts and technological approaches, always demanding rigor (the need to multiply controls and experimental rehearsals), interpretation and dissemination of results. The manner was a dialectic ping-pong until a consensus was reached. It was particularly efficient in coming to the “substantifique moelle” of the question to be examined. In this way, he undoubtedly left his indelible mark on each one of us. To draw a bit more of the shape of his “interior statue,” I shall now underline what I consider prominent traits a great leader must possess. In terms of human relationships, he had a friendly attitude and gave his colleagues freedom to undertake what they believe was important, which in his mind was the guarantee that they would give their best. When working in Orsay, he decided to cultivate some land located in front of the greenhouses and for many seasons I joined him working the soil and sweating a lot to finally collect nice tomatoes and some other vegetables. This story is to highlight a dimension of humility through the osmosis with the earth that PG had kept, while this was often forgotten by many colleagues of his rank. Finally, what best defines the personality of PG is the famous, oxymoron-like slogan “la force tranquille.” Overall, I recall the excellent atmosphere that prevailed in the laboratory and the many researchers that were fed on his philosophy can testify to the benefits of their encounter with PG. Among the impressive list of people cited above, most students that came to your laboratory are now working in teaching and research, worldwide, and keeping his lessons alive. In October 2019, Pierre sadly passed away “*Acta est fabula.*” Most of the citations concerning the work described in the text can be found in the following review articles: [Chollet, Vidal, and O’Leary \(1996\)](#), [Echevarria and Vidal \(2003\)](#), [Lepiniec, Thomas, and Vidal \(2003\)](#), and [Vidal and Chollet \(1997\)](#).

Acknowledgements: Hereafter are listed the researchers who have contributed to the writing, to some extent or extensively, of the PEPC story as described above. J. Maréchal, M-C. Rio, J. Gérard, C. Perrot-Rechenmann, M. Thomas, C. Créatin, L. Lepiniec, E. Keryer, R. Chen, J. Brulfert, T. Taybe, N. Bakrim, K. Tran, V-L. Bui, V. Pacquit, S. Rydz, N. Bakrim, M. Nhiri, C. Echevarria, J-N. Pierre, N. Giglioli-Guivarc'h, S. Coursol, J-L. Prieto, J. Grisvard, A. Gousset-Dupont, B. Leboutellier, P. Meimoun, and F. Ambard-Bretteville.

Thanks are due to researchers, teachers, technicians, and administrators from PG's laboratories and from the many (inter)national collaborators that we had over the years.

I am indebted to my long-time colleague, Michael Hodges, who corrected a certain number of our PEPC articles, including this one, a very good touchstone against which to refine interpretations, and who became a specialist of this plant enzyme and others.

2.1.2 Tatsuo Sugiyama's recollections

By *Tatsuo Sugiyama (Emeritus Professor of Nagoya University/ Emeritus researcher at RIKEN).*

As a researcher, Pierre has made great strides in studying the mechanisms and regulation of CO₂ fixation in higher plants and in the mechanism and regulation of nitrogen metabolism. His research trend was similar to mine. That's why the exchange with him has been going on for 40 years.

One of the most impressive moments in the exchange was a relationship of trust with his disciple, Dr. Akira Suzuki. That involved Bob Buchanan, Professor of University of California at Berkeley. That occurred in 1986. My lab was looking for an associate professor. Bob was aware of this and participated as a judge on Akira's PhD defense, and recommended to me that Akira fit the requirements. In response, I asked Akira directly to accept my invitation. He replied politely "Professor Pierre Gadal has nominated me as a public servant for my job. I am sorry but, unfortunately, I must refuse your kind invitation."

After that I had the opportunity to stay in Gadal's lab as a guest professor for a month. In the meantime, he struggled with the government to expand the research institute. So, I in fact conducted a collaboration work with Jean Vidal by doing the isolation of corn nuclei to be used for a run-on experiment. Anyhow, I was able to enjoy my stay enough.

It is unforgettable that this visit was triggered by Dr. Claude Cretin's participating in a collaborative work in my lab in Nagoya. Unfortunately, the young, talented researcher died after returning from Japan with fruitful results.

Pierre made a great contribution to the scientific collaboration between France and Japan in his later years. In 2004, a joint seminar was held in Nara, Japan's ancient capital, where he played the role of organizer on the French side. After the stay of Pierre and his wife in Japan, my wife and I began a private exchange with them and enjoyed the summer at Pierre's and his wife's villa in Brittany several times. Pierre then told us an interesting story about a wooden barrel in the corner of the living room of the villa. He told us, "The barrel was for my mother to kill and butcher the pig once a year, to salt the meat, and when I was a child, I stared in prayer so that mother would not fail to make valuable food." At other times, we drove to Bordeaux to help us deepen our understanding of French differentiation.

I will never forget the scientific achievements Pierre Gadal has left behind and our private warm-hearted exchange.

2.2 Redox regulation and the thioredoxin systems

As stated above P. Gadal oriented part of its research toward the study of the regulation of enzymes involved in carbon and nitrogen metabolisms. In line with the admonition of Ephraim Racker: "Do not waste clean thinking on dirty enzymes," the first efforts were initially concentrated on enzyme purification. The following testimonies explain the various steps that were used for this achievement.

2.2.1 Jean-Pierre Jacquot recollections

The early Nancy years [1975–1981] NADP-MDH activation, thioredoxin heterogeneity discovery.

In the Gadal lab, the earliest experiments concerning the redox regulation of chloroplast enzymes have dealt primarily with the elucidation of the NADP-malate dehydrogenase light activation process. It all started in 1975 at the University Henri Poincaré of Nancy where I performed the experimental part of my DEA (Master) study. Chloroplastic NADP-

malate dehydrogenase had been partially characterized in C_4 plants by Hal Hatch and colleagues and it was already known to be light activated, i.e., basically inactive in the dark and active in the light and responsive to thiol reagents as dithiothreitol (DTT) (Johnson & Hatch, 1970). At the time, the study of the light activation of chloroplast enzymes was already well underway in Berkeley where Bob Buchanan, Peter Schürmann and Ricardo Wolosiuk had obtained evidence for the existence of two regulatory proteins involved in the activation process of FBPase that they had baptized ARPa (later to become ferredoxin-thioredoxin reductase) and ARPb (later thioredoxin) required together with photoreduced ferredoxin (Buchanan, Schürmann, & Kalberer, 1971; Schürmann, Wolosiuk, Breazeale, & Buchanan, 1976). As was often the case at the time, I had two supervisors a senior professor Pierre Gadal and a more junior CNRS researcher Jean Vidal. The Gadal lab was just starting at the time, this was the third year after Pierre Gadal's arrival as a professor in Nancy after transferring from Toulouse. That year two other students started their Master project at the same time and we were distributed three different tasks, as I recall the other two dealt with glutamate synthetase and glutamate dehydrogenase. The project I was given was described as follows by Jean Vidal: I can't purify the NADP-MDH, the enzyme requires dithiothreitol to be active but the activity vanishes as soon as I try to purify it so there must be a protein factor required to activate it that I am losing along the purification. This was definitely visionary and it made sense in the light of the experiments done in Berkeley on the FBPase, but still I thought that the project was very risky because I could think of a zillion reasons explaining why an enzyme could be unstable in solution and thus I was not too happy to have been landed this project. In short, I thought it was too hypothetical and prone to failure. I couldn't have been more wrong as these initial experiments started in early 1976 would ultimately shape my career for the next 40 years or so. The first idea was to try to separate a protein factor (later Trx) required for the DTT-linked activation of NADP-MDH by gel filtration and to this effect we tried first to use Ultrogel ACA 44 or Sephadex G50 columns. At the time we had very little experience in protein purification and we started by using French bean leaves (a very poor biochemical material that turned immediately black when the polyphenols it contained oxidized, irrespective of the presence of antioxidants). As a result, the columns that we were using were quickly damaged and needed to be regenerated with cycles of base/acid treatments, but this also degraded the matrix polymers. On top of that we were using very small quantities of plant material in the first experiments (ca. 1/4th of a g of lyophilized material equivalent to ca 2 g fresh material). We would realize only much later that in order to purify reasonable quantities of highly purified proteins, it was actually necessary to use much higher quantities in the kg range for leaves. Nevertheless, we have very quickly determined that the NADP-MDH was likely present in the fractions eluting at the void volume and that "species" present in a fraction of lower molecular weight assisted the reactivation of the enzyme by DTT. Meanwhile a paper had described the purification of a LAF (light activating factor) of RubisCO from ethanol extracts (Wildner & Criddle, 1969) and we tried to adapt that with very limited success. At that time, it happened that Jean Vidal left for a congress and I was left on my own for a week or so. Somehow having read in the literature that some proteins are thermostable I tried my luck with the fraction putatively containing the regulatory protein and it was very exciting to discover that this regulatory activity was indeed thermostable at 80 °C for several minutes. Obviously, it offered the great perspective of separating easily the activating factor from the NADP-MDH by simply heating the sample. When Jean came back he was less than impressed and his first analysis was that the component in the activating fraction was probably not of protein nature as most proteins are not thermostable. After discussion, we set out at treating the activating factor with a protease called pronase and subsequently destroyed the pronase component by heating. In turn, we tested the activity of the protein fraction, which turned out to be totally destroyed. This clever experiment designed by Jean confirmed that the fraction that we had isolated indeed contained a protein. We could publish these results in December of the same year in *FEBS Letters* (Jacquot, Vidal, & Gadal, 1976). This was fortunate as Kagawa and Hatch published very similar results using a C_4 plant model instead. Interestingly their contribution indicated that the activating protein could also serve to deactivate, something that basically would not be explored further for another 40 years (Kagawa & Hatch, 1977). On the other hand, their contribution had not recognized the thermostability of the protein which would later help identify it as thioredoxin in the Buchanan and Holmgren labs. Although not extremely cited, our *FEBS Letters* paper in retrospect was very important as it opened the way to the study of redox regulation in plants in France.

Having obtained evidence for the existence of this regulatory protein it became of importance to verify that it was indeed also located in the chloroplast as the target enzyme and to generalize its existence to other photosynthetic organisms. By isolating chloroplasts, we could indeed demonstrate the chloroplastic localization of the regulatory proteins in spinach, a species renowned for being adequate for chloroplast isolation (it was also a much better biochemical material to work with compared to bean and much of the subsequent biochemical work in Berkeley, Neuchâtel and Nancy was done later with spinach) (Jacquot, Vidal, & Gadal, 1977). We then set out at studying the

presence of these regulatory proteins in Sorghum, a C₄ plant. In agreement with the results of Kagawa and Hatch we found that sorghum like corn indeed contained protein activating factors and we could show also that there were apparently multiple forms that could be chromatographically separated (Vidal, Jacquot, Membre, & Gadal, 1978). We confirmed a bit later the ubiquitous presence of the regulatory protein (thioredoxin) by studying its presence in the photosynthetic bacterium *Rhodospseudomonas capsulata* (Jacquot, Maudinas, & Gadal, 1979). Once again in this purple sulfur bacterium there was evidence for multiple forms of thioredoxin. In between Bob Buchanan, Ricardo Wolosiuk, Arne Holmgren and their colleagues had provided firm evidence that the protein factors were in fact thioredoxins (Holmgren, Buchanan, & Wolosiuk, 1977; Wolosiuk & Buchanan, 1977). A physiological significance for the existence of multiple thioredoxins in plants became clear after we started cooperating with Peter Schürmann in Neuchâtel. The idea was to compare the properties of the thioredoxins that were activating the NADP-MDH and those which activated fructose 1,6 bisphosphatase, the model enzyme in Berkeley and Neuchâtel. To this end I visited Peter in Neuchâtel during a week to learn about FBPase activation and measurement. This was basically the first scientific trip that I made in my career and I was enchanted both by Neuchâtel and its lake and Peter and his Swiss-German coolness efficiency and kindness. Most of all I learnt a lot about protein purification there, how to use larger quantities of leaves, scaling up the columns, etc. After this week of training, Peter handed me an FBPase sample which I accidentally dropped in the sink in Nancy after discarding the ice and recovered after the week-end at room temperature there. I would then do all the experiments with this sample which tells that FBPase is really a very robust enzyme... Anyway, when I probed the fractions obtained after gel filtration in low ionic strength I could show that FBPase and NADP-MDH were activated by different thioredoxin fractions, the one that activated FBPase eluting at molecular weights roughly twice the size of that activating the NADP-MDH. We could further purify these two thioredoxins by ion exchange chromatography. Jean Vidal and myself were more than a tiny bit happy about those results, these experiments were actually done at feverish pace as we were seeing real discovery unfolding there and we celebrated that very honorably. So we got in touch with Peter Schürmann either by snail mail (email and the internet did not exist) or by phone, but calling to another country was very expensive at the time so we probably relied on the post. Peter was actually less than excited as he had done replicate experiments in Neuchâtel with a partially purified NADP-MDH sample that I brought there. He had indeed obtained drastically different results in his parallel experiments, with NADP-MDH and FBPase being activated by the same fractions. Damned, all our hopes were shattered... So, we reviewed carefully the experimental conditions that we used, respectively, and Peter noticed that he was using a buffer with a much higher ionic strength for gel filtration compared to me and we hypothesized that this might be a reason for the difference. In any case we decided that I should visit again in Neuchâtel with the purified fractions exhibiting specificity versus the two target enzymes so that we could repeat the experiments together. The week that we spent there together working on the DTT activation of the enzymes was certainly one of the most exciting in my career. It started under nice auspices as in between Peter had repeated the chromatographies in low ionic strength buffer and had replicated our earlier results obtained in Nancy. We realized much later that thioredoxin f dimerizes in low ionic strength explaining the discrepancy. In short, we obtained evidence that leaves contained two different types of thioredoxin m and one type of thioredoxin f. In roots there was a single thioredoxin able to activate only the NADP-MDH (Jacquot, Vidal, Gadal, & Schürmann, 1978). This paper was definitely a second breakthrough for the Nancy group in the redox field and it was well received and granted us some more international recognition. The results were confirmed independently in Berkeley nearly at the same time (Wolosiuk, Crawford, & Buchanan, 1979). It was already mentioned that thioredoxin was known in other organisms as *E. coli* and human as a protein required for the regeneration of ribonucleotide reductase and hence deoxyribonucleotide and DNA synthesis. At the time colleagues working with those models had evidence for a single thioredoxin in there. I believe that they suspected for some time that our results were flawed as we had not used protease inhibitors, so there could be a protein degradation and generation of artifacts. While this was absolutely plausible, mounting evidence originating from plants indicated that the heterogeneous nature of the thioredoxins was a reality and then genome sequencing changed everything. Nevertheless, it has taken nearly another 20 years to obtain firm evidence for the existence of multiple thioredoxins and glutaredoxins in *E. coli* and other non-plant species (Lillig et al., 1999; (Miranda-Vizuete, Damdimopoulos, Gustafsson, & Spyrou, 1997) Miranda-Vizuete et al., 1997) but of course overall their contributions to the field were essential and they produced superb science.

So, going back to 1978 we then got interested by kinetic details of the activation and published a paper that featured a lot of mathematics describing the activation that did not excite too much the biologist community (Vidal, Jacquot, Gadal, & Vidal, 1978). At the same time, we then turned our attention to the more physiological light-dependent regulation. As Peter had purified the ferredoxin-thioredoxin reductase we now had all the components of the

ferredoxin-thioredoxin system in hand and could reconstitute the light system so I took my suitcase again and went back to Neuchâtel for yet another week. We could simplify the assay by working in sealed Eppendorf tubes under nitrogen a considerable improvement over the Warburg vessel used previously in Berkeley and Neuchâtel (Schürmann & Jacquot, 1979). This improved and lighter system confirmed the specificities of thioredoxins m and f. Together with Jean Vidal, a first light-dependent reconstituted system was then set up in Nancy including a crude version of ferredoxin-thioredoxin reductase and partially purified ferredoxin. We obtained also evidence for the in vivo light-dependent activation of the NADP-MDH by directly lysing chloroplasts into the reaction medium where they burst open because of osmotic shock (Vidal, Jacquot, & Gadal, 1980).

Meanwhile Pierre Gadal had attended the international photosynthesis congress in Reading in 1977 where he met Bob Buchanan for the first time. He came back with information that Bob was apparently a nice person to work with and an invitation for someone of our group for a sabbatical/post doc in Berkeley. At the time Jean Vidal and myself were both tenured CNRS researchers in the Gadal lab and both potential candidates for a 1-year visit. As Jean Vidal was senior he was naturally asked first but for a variety of reasons, mostly family-linked he declined to go. I couldn't believe my luck! At the time it was not a big problem for me and my family to go to Berkeley and I accepted enthusiastically as California was really a magnet culturally and intellectually. In Berkeley Bob asked me to purify the NADP-MDH from corn and I accepted although this would later create "border" problems in the Nancy lab that took a long time to heal. Anyway, the stay was very successful as I was able to purify the enzyme to homogeneity and study some of its kinetic properties (Jacquot, Buchanan, Martin, & Vidal, 1981). This was the same preparation that we used later in Nancy to generate specific antibodies against the enzyme that would in turn be used to isolate cDNAs from a λ gt11 library in Orsay by Claude Crétin. This sequence would then be used to generate an expression system in *E. coli* that allowed the structure-function study of that important enzyme.

And how did Pierre Gadal fit into that? Well to state it simply he was the one who boosted everything. First by selecting the subjects that he would like his lab to work on and that dealt essentially with carbon and nitrogen assimilation first. Although he was not a fantastic orator Pierre Gadal also had a knack for captivating and recruiting students of interest through his lectures that carried an unmistakable air of sincerity. Pierre Gadal was also a superb organizer and well connected in the world of French plant science. This helped the group created in Nancy expand notably through an association with CNRS which allowed the recruiting of five tenured researchers over a 10-year span. Pierre Gadal paid extreme attention to his younger colleagues and for example he accompanied me to Paris when I needed to interview for my CNRS job. This was very helpful as I had very little traveled before. Another key intervention is that he was the one who got initially in touch with Bob Buchanan and helped us connect with the big players in the field. A third one was the joint organization of the first thioredoxin congress by Pierre and Bob Buchanan in Berkeley in 1980, sponsored by NASA and CNRS. This was the first time I got in contact with Arne Holmgren the other king of that domain on the bacterial/mammal side. Many other congresses of that type would follow including one that I co-organized later in Roscoff in the Jacques Monod series. On a daily basis Pierre was at that time following our research projects very closely, the magic sentence being every Friday "So how has it been, got new results?" Also, he has been throughout his career constantly pushing us to publish our results in peer-reviewed journals and in English, which, in the middle of the 70s was not yet so common in France. The latter section dealing with the work in Orsay will detail further his organization skills and scientific visions that proved essential for turning later to molecular biology techniques. Still, on a more personal basis it was fun to work in the Gadal's group, we did many things together, much sports including jogging some biking, an occasional odd football tournament in Nayemont-les-Fosses and quite a bit of ping-pong as we had a table next adjacent to the lab. Among the contests that we were having were vertical jump tests where we had to jump as high as possible and we recorded the results with blue chalk on a beam hanging down the ceiling. From that time, I can indeed testify that white men can't jump... Anyway, our performances were pretty much related to our heights, Jean being the most altitudinally challenged, experiencing the most difficulties. Pierre was substantially taller than most of us and when he saw us doing that he came and touched the ceiling on his first try and left with a shrug meaning "this type of contest is too easy for me." Humiliated we were, thank you for this one Pierre. Another of the contests that we had routinely was chest band pull with Jean much more at ease there with his shorter limbs (besides he was very strong anyway)... We were also occasionally testing ourselves at the arm wrestling contest, Jean Vidal was very good at it but nobody wanted to play that game with Pierre Gadal worried that we were that he would destroy our shoulders...

Pierre Gadai was in general a rather reserved person never communicating on the informal “tu” basis but always maintaining the more formal “vous.” Nevertheless, Pierre himself played ping-pong quite enthusiastically and I remember a Saturday afternoon where he was teaming in a double with Jean Vidal against myself and Bertrand Hirel a game that became so serious that one of us (BH) ended up with a broken tooth (from a racket incident and not from a fight...). Except for incidents of that type working there was good old fun and Pierre was clever enough to mix professionalism with sort of a family-like atmosphere. I believe that was a big success for this group, it provided enthusiasm (it was kind of standard to work 10 h a day including week-ends when the research adrenaline kicked in) and results followed.

The later Orsay years [1981–1997] Protein sequence determinations, gene cloning, site-directed mutagenesis and structural output.

In 1981 Pierre Gadai applied successfully for a professor position in Orsay near Paris and asked his staff whether they would agree to move there as the CNRS-associated lab was to be transferred at the University of Paris-Sud. Essentially all of the colleagues active in research agreed to the move and I was one of them. Moving to Orsay was accompanied by a reorganization of the team and it was there that the nucleus of the redox team took form with the beginning of a long-standing cooperation with Myroslawa Miginiac-Maslow and later Paulette Decottignies. Together with Myroslawa and Michel Droux we started by isolating ferredoxin-thioredoxin reductase and refining and simplifying the light-dependent ferredoxin-thioredoxin system. This sequence of events will be described in more detail in the recollection of Myroslawa and Michel. One decisive technical advance was the arrival of Paulette Decottignies and Pierre Le Maréchal and their know-how in protein sequencing with the help of Jean-Marie Schmitter initially. This more or less coincided with us turning to a new biochemical model *Chlamydomonas reinhardtii* referred to later in this recollection as Chlamy (Huppe, de Lamotte, Jacquot, & Buchanan, 1990). Initially we thought that Chlamy would be a fantastic tool for generating mutants of the FTR system but this was not to be the case as the genes that we were interested in were nuclear ones and gene targeting in Chlamy worked well but only for chloroplastic genes at the time. Anyway, Chlamy turned out to be a beautiful biochemical material to work with, generating enzyme extracts much cleaner than what we got from spinach, corn or bean. This property combined with the fact that the genome of Chlamy was more simple than the one of land plants (a property that was not known in the early 90s) allowed us (after many efforts and a lot of work) to finally obtain substantial amounts of pure ferredoxin, ferredoxin-NADP reductase, chloroplastic thioredoxin m and cytosolic thioredoxin h. Paulette and Pierre were subsequently able to cut the proteins in pieces with the help of various proteases including trypsin, chymotrypsin and SV3 and to isolate and purify the peptides by HPLC. Edman sequencing of the intact proteins straight from the N-terminus and also of the various peptides was rather tedious and needed an iron organization but this was fine for those chemist-oriented researchers and they were able to fully sequence those four proteins (they range in size from 94 to 320 amino acids per polypeptide) (Decottignies et al., 1990; Decottignies, Schmitter, Dutka, Jacquot, & Miginiac-Maslow, 1991, Decottignies, Le Maréchal, Jacquot, Schmitter, & Gadai, 1995; Schmitter et al., 1988). In parallel to those efforts, Claude Crétin had isolated a cDNA coding for sorghum NADP-MDH by screening a λ gt11 expression library with antibodies and I had been able together with Eliane Keryer to introduce it into an expression plasmid and to generate some recombinant protein in *E. coli*. Actually seeing that additional tiny protein band in the *E. coli* extracts was one of the most memorable achievements in my scientific life. This observation was also accompanied by the appearance of a new oxalacetate reducing activity, NADP-dependent, and requiring reduction to be active in the *E. coli* extracts and that was definitely the cherry on the cake (Jacquot et al., 1991). Champagne on that day! Meanwhile we were also active on other fronts learning molecular biology techniques and site-directed mutagenesis. We started with Frederic de Lamotte and Philippe Minard by engineering a mutation on the *E. coli* thioredoxin gene (D61N) that allowed the new protein to acquire a thioredoxin f-like property (de Lamotte-Guéry et al., 1991). In parallel, together with Mariana Stein and John Rogers we started isolating by PCR the cDNA fragments corresponding to the coding sequences of ferredoxin and thioredoxin m (called Ch2 then), based on the previously determined protein sequences (Jacquot, Stein, Hodges, & Miginiac-Maslow, 1992; Rogers et al., 1992). This was facilitated by using preferential codons for the Chlamy nuclear genes that heavily favor those ending with G or C, A and T being in general not used in this organism. We could thus limit the number of degeneracies in the forward and reverse oligonucleotides and this proved to be crucial for the success of this PCR cloning. The initial fragments that we had generated concerned only the mature part of the polypeptides generated after cleavage occurs following import into chloroplasts. Nevertheless, in a later step we were able using either antibodies or the ³²P labeled PCR fragments to isolate full-length cDNA clones from λ gt11 libraries that featured the 5' and 3' UTR and also the sequence coding for the transit peptide needed for import. In a

subsequent step, those fragments were labeled to isolate the corresponding clones from a genomic library together with the one coding for thioredoxin h (Stein et al., 1995; Stein, Chedozeau, & Jacquot, 1996; Stein, Jacquot, & Miginiac-Maslow, 1993).

So in a few years we had gone a long way to determine the primary structure of several of the enzymes of the ferredoxin-thioredoxin system. This was very quickly followed by developments that led to the elucidation of 3D structures of those proteins. The initial work with the NADP-MDH had gotten us introduced to genetic engineering and the production of recombinant proteins, and rather quickly we adopted a new type of expression plasmid from the pET series that was a lot more efficient. This has allowed us to produce now massive amounts of multiple thioredoxins including pea thioredoxin f with Michael Hodges, NADPH-thioredoxin reductase together with Yves Meyer in Perpignan, FBPase with Lopez-Gorge and Anna Chueca in Spain that would then lead to solving several 3D structures (Hodges et al., 1994; Jacquot et al., 1994, 1995; Lopez-Jaramillo et al., 1997; Rivera-Madrid et al., 1995). A whole new area of research opened after we shifted to that new expression tool and we were able to perform site-directed mutagenesis to determine which cysteines were essential for the NADP-MDH regulation (see the description of **Q9** Myroslawa for that) but also for FBPase (Jacquot et al., 1997). Likewise, we could determine which acidic residues govern the tight association between ferredoxin and ferredoxin-thioredoxin reductase (Jacquot, Lopez-Jaramillo, et al., 1997).

3D structures of full-length enzymes have been then rapidly produced by X-ray crystallography for NADPH-thioredoxin reductase with Hans Eklund and Shaodong Dai in Uppsala (Dai et al., 1996) and FBPase with Jacqueline Cherfils in Gif sur Yvette (Chiadmi, Navaza, Miginiac-Maslow, Jacquot, & Cherfils, 1999). Using NMR and through a very fruitful cooperation with Jean-Marc Lancelin we have been able to solve structures for thioredoxin m, thioredoxin h as well as for transit peptides required for protein translocation (Lancelin, Stein, & Jacquot, 1993; Lancelin et al., **Q10** 1994; Mittard et al., 1995).

This describes essentially the main output of my stint in Orsay, but there were also quite a number of side projects to which we participated. Among others they include the discovery of thioredoxin in human (Jacquot et al., 1990; Wollmann et al., 1988), the description of thioredoxins in *Dictyostelium discoideum* (Wetterauer, Jacquot, & Véron, 1992) and the interactions of ferredoxin with cellular components other than FTR, such as PSI (Fischer, Hippler, Sétif, **Q11** Jacquot, & Rochaix, 1998) and glutamate synthase (García-Sánchez, Gotor, Jacquot, & Vega, 2000) (García-Sánchez et al., 2000).

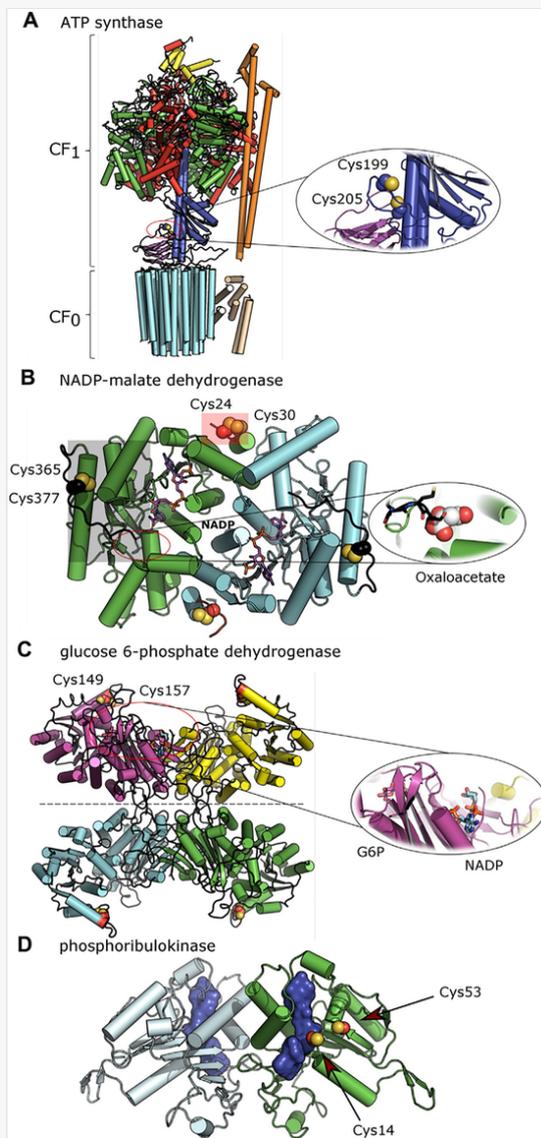
What about the social life in the Gadad Orsay lab? Well it was equally fun compared to Nancy, our interests moved to playing bilboquet, lots of biking and jogging in the woods surrounding the beautiful Orsay campus. We also initiated a new one, rope jump, at which we became pretty good but on one occasion Keishiro Wada visiting us stunned us by showing how a guy close to 60 could do it squatting on his haunches, a very difficult thing to do. Another of those humiliations there... Intellectually we kept stimulating ourselves playing a French version of Trivial Pursuit, Frédéric was very good in the pink category there (the vainest of them all) ... Of course, we still played quite a bit of ping-pong as we had three tennis tables in the basement of the building underneath the greenhouse. Pierre Gadad then in his 50s was still very keen on playing and we could communicate a lot that way as otherwise he became very busy with administrative duties (he was actually a superb organizer of science). Nevertheless, the best tennis table player was by far Stéphane Lemaire as he handily won the first tournament of our Institute. Also, with Frederic de Lamotte it was fun to try to pull a Nelson (double Nelson in French) on one another especially when the colleague was pipetting and could hardly defend himself. This slowed Frédéric a bit for the cloning of his mutants but he was so bright and dedicated that he succeeded anyway. As supervisors with Philippe Minard we had to kick his ass a couple of times to further motivate him, not that we enjoyed that too much but it worked in the end.

In 1997 after more than 20 years as a CNRS researcher I moved to another position as a Professor at University of Nancy I later to become Université de Lorraine. The work that was subsequently done in Nancy focused on other redox aspects including the study of thiol peroxidases and their relations to thioredoxin and glutaredoxin, and more recently the assembly of iron sulfur clusters, a process in which glutaredoxin is heavily involved. All of the work that was done in this later step of my career would not have been possible without the Orsay experience where we learnt how to combine biochemistry with molecular biology, became acquainted to PCR and had the first access to genomics data on *Arabidopsis thaliana*. Fortunately, the enthusiasm for research that was the trademark of the Gadad's lab has been transferred to the Nancy redox group later headed by Nicolas Rouhier and the results have followed.

Biology is a science that develops incredibly rapidly especially at the technical level. Of course with the tools generated today what we had done in the 90s could be done in a matter of weeks or months as genomes are now sequenced and we do not have to chase for sequences any more with those “primitive” tools. Most of this was initiated before genomics and PCR and therefore the rhythm of scientific production was quite slow compared to today's output. At first restriction enzymes were scarce, genomics and proteomics inexistent, DNA sequencing had to be done manually with the Sanger method and 35S using long acrylamide gels that were a pain to prepare, etc. In retrospect, was that all in vain? Well there are some facts that remain of high interest even after all these new developments. For instance, the route that we had to follow included the isolation of proteins before we could isolate the genes. Taking Chlamy ferredoxin as an example it is one of the few instances where we know exactly where the cleavage or the preprotein takes place as we know the real N-terminus of the chloroplastic processed protein. Nowadays people “guess” where the cleavage is supposed to take place by bioinformatics but this is basically never tested experimentally (although proteomics can now provide data about that). Incidentally, when you produce a recombinant protein, cleaving the presequence in the right position can be very important for the production of a soluble recombinant protein. Another example of the interest of this approach is ferredoxin-NADP reductase. When we confronted the results of protein sequencing to those of DNA sequencing it became clear that this protein is in vivo methylated at three positions on Lysine residues, a property that we could never have deduced from the sole DNA sequence (Decottignies et al., 1995). Besides, and more importantly our work has given insight on how disulfide bonds put a physical strain on regulatory enzymes and how reduction relaxes this strain and allows them to shift into catalytically competent conformations. A couple of Figs. 1 and 2 extracted from a rather recent review paint the molecular details for many of the redox-regulatory enzymes connected to photosynthesis (Güttele et al., 2017). A common feature to all these enzymes is that their redox-regulatory site is in general distant from the active site and the regulatory cysteines are not involved in catalysis. Yet they influence at a distance the opening of active sites and their accessibility to substrates. Of course, this is crucial for the regulation of photosynthesis and probably agronomical yields. A final comment is that the raw data of genomics have little meaning as long as you don't know what the function of a given gene/protein is and that is best obtained from biochemical experience. So, in this sense all the work generated in the Holmgren lab in Sweden, the Buchanan lab in California and, hopefully, to some extent our lab in Nancy/Orsay/Nancy was not futile after all.

alt-text: Fig. 1

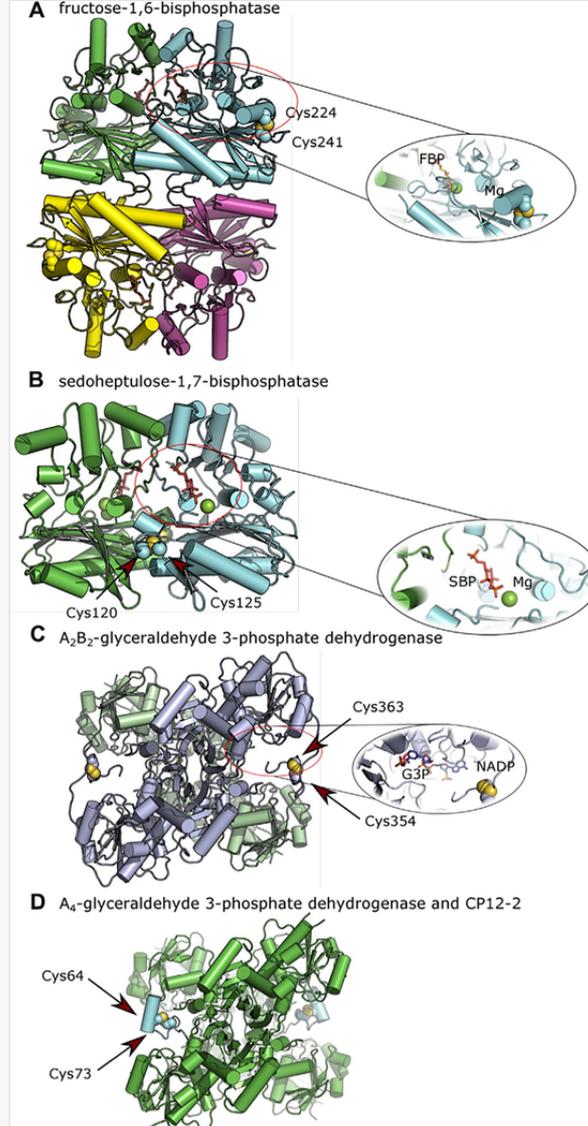
Fig. 1



3D structures and models of redox regulated chloroplastic enzymes. (A) CF1 ATPase, (B) NADP-malate dehydrogenase, (C) glucose 6 phosphate dehydrogenase, and (D) phosphoribulokinase. Cys labeled 30 of NADP-MDH is, in fact, Cys 29. From Gütle, D.D., Roret, T., Hecker, A., Reski, R., & Jacquot, J.P. (2017). Dithiol disulphide exchange in redox regulation of chloroplast enzymes in response to evolutionary and structural constraints. *Plant Science* 255, 1–11..

alt-text: Fig. 2

Fig. 2



3D structures and models of redox regulated chloroplastic enzymes. (A) FBPase, (B) SBPase, (C) GAPDH A2B2, and (D) GAPDH A4 with CP12.

From Gütle, D.D., Roret, T., Hecker, A., Reski, R., & Jacquot, J.P. (2017). Dithiol disulphide exchange in redox regulation of chloroplast enzymes in response to evolutionary and structural constraints. *Plant Science* 255, 1–11..

2.2.2 Frédéric de Lamotte's recollections of the Orsay time [1985–1990]

Pierre Gadal was my professor at University of Paris-Sud, Orsay. I attended his Plant Physiology courses during my bachelor and master. I also performed the experimental part of my master in his lab, then my PhD research under the brilliant supervision of Jean-Pierre Jacquot. Pierre Gadal was an unusual professor. During his lectures, I had the impression of being in front of Science, of glimpsing the “big picture.” I felt like I was intelligent (even though I was disappointed about it more than once afterwards...).

When I joined the group, the main elements of the Redox cascade had already been identified and purified in higher plants by “the first generation” as Jean-Pierre described earlier. The next step was to focus on one organism, *Chlamydomonas reinhardtii* referred to later as *Chlamydomonas*.

The choice was guided by several reasons. At that time, it was not yet established that the FTR system was present in all photosynthetic organisms. In addition, *Chlamydomonas*, a unicellular eukaryotic organism, was easily amenable to microbiological techniques and its genetics was known. Moreover, we were hoping to finally have an organism that could supply all the bricks for the redox cascade.

My first research work was to set up a culture system and thanks to J-P. ingenuity, my naive enthusiasm and the benevolence of P. Gadad, we were able together with Heather Huppe to grow *Chlamydomonas* in big 25 L glass bottles, with a large magnetic bar rotating at the bottom, pressurized air bubbling from the top and neon light shining from the side, sitting on the floor of the main lab. This particular noise and light would accompany me throughout my thesis.

The second part of my work was to learn how to harvest and concentrate the cells, then extract and purify the proteins. Everything went fine thanks to the strong support and expertise of Paulette and Myroslawa. Ferredoxin was swiftly purified and sequenced. We were not able to obtain a highly purified form of the MDH but the fraction was active enough to conduct light activation experiments Two thioredoxins were identified as well as the FTR. The sequence of the two thioredoxins was determined by Paulette. One was clearly an m type and the other one was later identified as an h type. These first results were published in 1988 (Schmitter et al., 1988) and 1990 (Huppe et al., 1990). This study made clear that the FTR system was ubiquitous in all photosynthetic organisms, reinforcing its cornerstone position and the interest of its study. At the time, I wasn't really surprised at how easy it was, being successful after just one or two attempts; it was only later that I realized how lucky I had been.

During my PhD work, we then focused on thioredoxins. In the redox cascade transmitting electrons from the photosystems to the activation site of the target enzymes, thioredoxins exhibit heterogeneity with the m, f and h types known at the time. We wanted to understand what carries the specificity of each of these proteins. The sequence of the *Chlamydomonas* type m showed quite a lot of similarity with the thioredoxin from *E. coli* (TrxA). As, unlike the ones of our green algae, the *trxA* gene was available, we decided to use the bacterial protein for this study. Here again, I was lucky as, in Janine Yon-Khan lab, next door, Philippe Minard was just coming back from his post doc in a UK lab where he worked on the first site-directed mutagenesis experiments. This was actually initiated after Pierre Gadad's suggestion who came back from a meeting thrilled by a presentation and telling us: you have to do site-directed mutagenesis, it's the future.

As enthusiastic and intrepid as ever, we have embarked on the development of an overproduction system for thioredoxin in a bacterial system. We cloned the *trxA* gene into a high copy number plasmid and rapidly set up conditions for the production and the purification of more than 60 mg of purified thioredoxin per liter of culture. That yield was considered as gigantic at the time. The purified protein was able to mimic the plant thioredoxin m behavior in the photomodulation system.

The next step was closer to a giant leap rather than a regular step as it was to make site-directed mutagenesis in an era before PCR was available... The first oligonucleotides carrying the mismatch were made on a (very) first generation of oligonucleotides synthesizer which would require to push four syringes (for the four nucleotides) in the correct sequence. The purified oligos were hybridized on a single stranded DNA (we needed to purify it from phage M13mp19, and that was very difficult, it worked only when the phage was willing) and the double stranded DNA was synthesized in vitro. The yield of this method (5%) was a real drawback until Amersham and Bio-Rad came with some kits. The price was extremely expensive but, once again, P. Gadad provided us a strong support, pushing forward. This new kit allowed me to produce my first mutants. The choice of the amino acids to be modified was made on the basis of the available biochemical data and a comparison of the different published primary structures. The mutants fell into two categories corresponding to the folding of the protein and the putative interactions with the target proteins. It should be noted that it was very advantageous to have the 3D structure of the *E. coli* TRX in order to localize each amino acid, and thus be able to refine the choices.

The first mutant was named D61N, meaning that Aspartic acid number 61 was replaced by its neutral equivalent Asparagine. With the exception of the thioredoxin f, all thioredoxins known at the time had a charged residue at position 61. Interestingly, thioredoxin f is the only plant thioredoxin that forms a stable complex with its target enzyme. Would it be possible to shift the behavior of the thioredoxin from the m type to the f type by changing just one residue?

We purified the modified protein and its wild-type counterpart. The recombinant proteins were analyzed with a whole set of tests, physicochemically, biophysically and catalytically. HPLC, IEF and native electrophoresis confirmed the charge difference compared to the wild type. Fluorescence spectra and circular dichroism detected no differences, as expected.

Three reduction sources were used: DTT, NADPH with *E. coli* NADPH-TRX reductase (NTR), and spinach FTR. The reduced thioredoxins were used to reduce four targets: DTNB (dithiobis nitrobenzoic acid), insulin, NADPH with NADPH-TRX reductase from corn (thioredoxin *m* target) and FBPase from spinach (thioredoxin *f* target). These tests revealed differences in efficiency in favor of the mutant for FBPase activation when DTT provides the reducing power. This confirms the hypothesis that the charge carried by Asp61 plays a detrimental role in the interaction with FBPase. A less predictable result was the greater efficiency of the mutant in activating chloroplastic enzymes (NADP-MDH and FBPase) in the reconstituted system. This improved efficacy is explained by an improvement in the interaction between TRX and FTR. This work was published in 1991 ([de Lamotte-Guéry et al., 1991](#)).

The second one was named P40A, in which the Proline was replaced by an Alanine. This proline, found in all known thioredoxins, is located in the middle of the $\alpha 2$ helix. This position can have a significant impact on the structure of the protein and modify its thermostability.

The enzymatic behavior of P40A and the wild protein measured in the same conditions appeared identical. With the same tests used for D61N, only the fluorescence analysis showed a difference. At that time, it was impossible for us to interpret these results. Later, I moved to Montpellier, where I had the opportunity to collaborate with the Centre de Biochimie Structurale for the study of wheat thioredoxins. We built a molecular model of P40 A in order to interpret my observations.

P40A slightly differs from WT, the RMSD is 1.5 Å. The major difference is the helix $\alpha 2$ which is less bent, the angle formed by this helix shifting from 59° to 13°. This straightening induces a displacement of the active site by 6 Å without modifying its structure. The hydrophobic contacts involving TRP31 and the loop between $\beta 2$ and $\alpha 3$ have been removed. W31 becomes accessible to the solvent, which explains the fluorometry results. These calculations also predicted that P40A should be less stable and more hydrophobic than WT. This was verified by hydrophobic interaction chromatography and denaturation with Guanidine Chloride followed by fluorescence at 340 nm; ΔG_0 went from 8.9 down to 6 kcal/mol.

Thus, contrary to our hypothesis, the proline that bends the helix $\alpha 2$ has no overall destabilizing effect. This can be explained by an unfavorable local effect on the helix and a more important favorable effect by optimization of the hydrophobic contacts. This was finally published in 1997 ([de Lamotte et al., 1997](#)). We can see here that a more precise and better argued answer has been brought by a multidisciplinary work. This kind of approach has been, since then, my routine.

On a more personal ground, I recall the following. By the end of my PhD work, I applied for a Post-Doctoral fellowship with a research project to EMBO. I was asked by EMBO to visit a member of their scientific board for an “informal discussion” about my project. The person I should discuss with was based in Warwick and the only practical way to get there was by airplane. The round-trip ticket was pretty expensive (about 1 month of my graduate student salary). As soon as Jean-Pierre informed Pierre Gadad, he offered to cover the cost of the ticket with the lab's budget. I was touched by this act of kindness, all the more so as it was about my own future and the repercussions of this event were very limited for Pierre Gadad and his laboratory. This is a fine example of the altruism that Pr. Gadad could show.

There is a common metaphor about scientists being dwarfs standing on the shoulder of a giant. Pierre Gadad was such a giant, a friendly giant. His shoulders were pretty high but hopefully I was helped to reach them by (in order of size more than in order of importance...) Paulette, Myroslawa and Jean-Pierre. My time spent in P. Gadad's lab had a deep and long-lasting influence on what I have become.

Without ever approaching his scientific radiance, I tried to copy his benevolence and love of people. In this, one can say that Pierre Gadad has had a major influence on my life as a researcher.

Side note. I would like to stress one point. I won, without a possible doubt, the Nelson contest with Jean-Pierre...

2.2.3 Stéphane Lemaire's recollections

In the 90s, Pierre Gadad was the director of the master school (at that time named DEA) dedicated to *Plant Molecular and Cellular Biology* in Orsay. I had studied genetics and molecular biology during my undergraduate studies and I was fascinated by plant systems. It rapidly became clear to me that integrating Pierre's master school was the most interesting path for me. As this path was very competitive, I thought that I would increase my chances of being selected

if I could join the group of Pierre Gadal for a short internship during the summer of 1994. I therefore contacted J-P. Jacquot and I was very lucky to be selected for this internship during which I discovered the true essence of scientific research. I recall the stimulating and friendly environment of the Gadal lab with the mentoring of Jean-Pierre. The aim of this internship was to perform site-directed mutagenesis on FBPase to confirm the exact cysteines involved in its thioredoxin-dependent activation. Although M13-based site-directed mutagenesis had been established in the group, Jean-Pierre wanted to test a new method based on PCR. These experiments were successful although I did not realize at that time that it was the first PCR-based mutagenesis performed in the lab. I clearly remember reading and writing by hand with Jean-Pierre the autoradiography of the 35S Edman sequencing gel (on a dedicated illuminated desk located in Jean Vidal's office) and our joint excitement when we confirmed that the mutagenesis had perfectly worked as expected. We could then describe the molecular mechanism of activation of FBPase by thioredoxin (Jacquot et al., 1995, 1997). After this internship I could join the master school program where I embraced molecular biology through the guidance of Pierre Gadal who was advocating, as mentioned by Jean-Pierre, the power of molecular biology for plant biology research. I joined again the lab for my Master internship where I studied the structural determinants of thioredoxin thermal stability with Jean-Pierre taking advantage of PCR site-directed mutagenesis. I could not immediately start a PhD since I had to join the military forces for 10 months, as mandatory at that time in France. I was very worried that being away from science and the lab for nearly 1 year would compromise my chances to be granted a PhD fellowship. Pierre Gadal had a decisive role for me during this period since he allowed me, thanks to his network, to obtain a military position near the lab to be able to keep in touch. He also played a decisive role to allow me to finally obtain a fellowship to initiate a PhD under the supervision of Jean-Pierre Jacquot in 1996. My work was the direct continuation of the work initiated in the Gadal group on thioredoxins and was aimed at pursuing the biochemical and structural analyses on thioredoxins but also growing *Chlamydomonas* under different light regimes to monitor the changes in expression of thioredoxin and related genes (Lemaire, Hours, et al., 1999; Lemaire, Keryer, et al., 1999; Lemaire et al., 2000). After my postdoc, I was hired by the CNRS in the same group, then led by Myroslawa Miginiac-Maslow. In 2010, I created my own group in Sorbonne University. This group is like many others a heritage of the Gadal school of thinking. Pierre has followed my career with a strong interest and was always very keen to discuss science, science policy and to provide his advices and recommendations. Today, we are still exploring redox regulation and the Calvin-Benson cycle in *Chlamydomonas* (Michelet et al., 2013). Recently, we embraced the emerging field of synthetic biology (Crozet et al., 2018; Vavitsas et al., 2019) and I am advocating to my colleagues and students the importance of these new approaches. This reminds me the pioneering view of Pierre who was advocating the importance to develop molecular biology in the plant research community. Throughout all these years under the guidance of Pierre and his mentees, Jean-Pierre and Myroslawa, I learned not only the passion of scientific research but maybe more importantly the human adventure of the scientific enterprise. Beyond his enthusiasm for science, Pierre is probably one of the most kind, gentle and caring scientist I met. He always took time to discuss and listen to the people around him, whatever their social status could be. Finally, Pierre Gadal has clearly shaped the scientist I am today. I will continue to humbly walk in his footsteps to perpetuate his heritage and I am very proud to be standing on the shoulders of this giant.

Q12

2.3 Light activation via ferredoxin-thioredoxin reductase: The NADP-malate dehydrogenase model

2.3.1 Myroslawa Miginiac-Maslow: Recollection from 1981 until Pierre Gadal's retirement in 2003 and beyond

In 1981, Pierre Gadal obtained a full professor position in Orsay, in replacement of Alexis Moyses who was retiring. Remarkably, almost all the members of the Nancy team followed him, despite family ties, witnessing the strong charisma of their leader. Several researchers from the former Moyses lab moved to other labs, but a few (including myself) remained and joined the new lab. At that time, many studies dealt with the ideal ATP/NADPH ratio necessary for optimal photosynthetic CO₂ fixation. My own results showed that this ratio could vary according to the ambient conditions, suggesting that linear and cyclic electron flow could occur simultaneously. Another intriguing observation was the stimulation of CO₂ fixation activities in some intact chloroplast preparations by Antimycin A, an inhibitor of cyclic electron flow, but also by some uncouplers, activating the electron flow (Miginiac-Maslow & Hoarau, 1975). In line with these results, evidence was obtained in the lab (Champigny & Bismuth, 1976) that these inhibitors enhanced the activity of several Calvin cycle enzymes measured in chloroplast stroma, suggesting the involvement of the reducing power in these phenomena. The arrival of persons working on enzyme redox regulation reactivated my

interest in the problem, suggesting that the ideal ATP/NADPH ratio should take into account the amount of reducing power needed for enzyme activation. Then we set up a collaboration with J-P. Jacquot, with the blessing of Pierre Gadal, to determine the light saturation values of the activation of NADP-MDH in a reconstituted light activation system. This was made possible by the availability, in my former lab, of a Warburg apparatus I had equipped with strong light bulbs, and a series of neutral filters allowing to establish light saturation curves. As a prerequisite to these experiments, we optimized a reconstituted light activation system, by purifying all of its components (ferredoxin, ferredoxin-thioredoxin reductase and the target enzyme) to homogeneity, and using thylakoids isolated from pea leaves, much more stable than those from spinach, which could be stored in a frozen form (Jacquot, Droux, Miginiac-Maslow, Joly, & Gadal, 1984). This rather trivial modification allowed ending a controversy between Bob Buchanan (using thylakoids from spinach) and Louise Anderson (using thylakoids from peas), the former presenting Trx as the activating factor, and the latter proposing a membrane-bound activating factor named LEM. In fact, with once washed pea thylakoids, we could perform enzyme activation without adding Trx, whereas, with twice washed pea thylakoids (one of the wash steps including NaCl) the addition of Trx was absolutely necessary. Thus, apparently, in peas, part of the Trx was membrane-bound. The purification of the protein components benefitted from the input of Michel Droux, the first of a series of clever and very efficient PhD students selected by the DEA committee headed by Pierre Gadal, who joined our group and achieved the full purification and characterization of FTR (Droux et al., 1987). At that time, there was some controversy concerning the chemical structure of FTR, in particular the presence of an iron-sulfur cluster. Using a final purification step with an affinity chromatography on a ferredoxin Sepharose column (and starting with 10 kg of spinach leaves) we could obtain a pure FTR preparation displaying a 4-iron-4-sulfur cluster but also a disulfide bridge, a feature that had never been detected before. We established also the sequence of events leading to the activation of NADP-MDH, namely sequential reduction of ferredoxin, FTR, Trx, and finally target enzyme (Droux et al., 1987). This important contribution, started during Michel's PhD, was finalized during Michel's post-doctoral stay in the lab of Bob Buchanan, in Berkeley, where the thiol groups of the different proteins involved in the activation process could be visualized using a new fluorescent agent, monobromo bimane (mBBBr). The molecular mechanism of action of FTR was later elucidated in the laboratory of Peter Schürmann, who solved also its 3D structure in collaboration with the laboratory of Hans Eklund in Uppsala, Sweden (Dai, Schwendtmayer, Schürmann, Ramaswamy, & Eklund, 2000).

The light activation curves showed that enzyme activation was saturated at low light intensity (10 W m^{-2} under nitrogen, 50 under air, where some reoxidation certainly occurred, vs 240 W m^{-2} for reduction of NADP). Thus this mechanism was not very energy-demanding (Miginiac-Maslow, Jacquot, & Droux, 1985). Having in hand the purified components of the system, we could titrate the cysteine thiols appearing on the activated enzymes, by using a C14-labeled alkylating agent, generally iodoacetamide. Then we could compare the reduction efficiencies of NADPH and Trx and conclude that NADP acted like a competitive inhibitor of Trx reduction, i.e. was favored in the system. We could also observe that the reduction of NADP-MDH was in fact very fast, as fast as the reduction of Trx, but that the activation of the enzyme lagged behind, taking about 10 min to reach full activity, suggesting that a slow conformational change followed the reduction step (Miginiac-Maslow, Decottignies, Jacquot, & Gadal, 1990).

Meanwhile, Gadal's lab attracted new people that joined our group: Paulette Decottignies and her husband, Pierre Le Maréchal, organic chemists working previously in a neighboring biochemistry lab. Pierre Gadal considered that their integration would bring new technical skills to the lab. Indeed their presence prompted us to try to analyze further, at the molecular level, the modifications occurring on NADP-MDH upon activation. The enzyme was incubated in a reconstituted light activation system under saturating white light. Then, the cysteines were alkylated with radioactive iodoacetate, the components separated by HPLC and the N-terminus of NADP-MDH sequenced in collaboration with J-M. Schmitter (Biochemistry lab of Ecole Polytechnique). Two labeled cysteines were found in the N-terminal part of the enzyme. None was found upon incubation in the dark. Then we considered that we had found the regulatory disulfide of NADP-MDH (Decottignies et al., 1988). In parallel with their contribution to the study of NADP-MDH, Paulette Decottignies and Pierre Le Maréchal determined the complete amino acid sequences of several proteins involved in redox metabolism, such as ferredoxin-NADP reductase and ferredoxin from *Chlamydomonas* (Decottignies et al., 1995; Rogers et al., 1992). The subsequent acquisition of a protein sequencer by the lab, thanks to funds obtained by Pierre Gadal, helped them to develop this important analytical tool for further studies.

When sorghum NADP-MDH was cloned and its cDNA sequenced (Crétin et al., 1988, 1990) we realized that each monomer of this dimeric enzyme possessed eight cysteine residues, strictly conserved in all the light-activatable NADP-

MDH, but also two conserved sequence extensions compared to permanently active NAD-MDH. The two cysteines putatively involved in the activation process were located in the N-terminal extension (Cys 64 and 69 in the full-length sequence, comprising the transit peptide of this nuclear-encoded chloroplastic protein, corresponding to Cys 24 and 29 of the mature protein). There was also one Cys located in a C-terminal extension, with another cysteine located nearby. An expression and recombinant protein production in *E. coli* having been set up, and after showing that the recombinant protein displayed the same regulatory properties as the enzyme isolated from sorghum leaves (Jacquot et al., 1991), we decided to try to confirm the results obtained by chemical analyses by using site-directed mutagenesis. Emmanuelle Issakidis, a new PhD student, who acquired a strong expertise in molecular biology, compared various recombinant protein expression systems and developed an efficient site-directed mutagenesis protocol using a pET-type plasmid. The first mutations she created targeted the two N-terminal cysteines previously identified, which were substituted individually or simultaneously for either alanines or serines. We were expecting to obtain fully active, redox-insensitive enzymes. The first activity tests brought a full disappointment: our mutated NADP-MDHs still required reduced Trx to reach full activity (Issakidis et al., 1992). However, looking more precisely at the activation kinetics, we noticed that enzyme activation was very fast, as fast as the reduction of Trx we had observed before. Clearly, there was no lag anymore between reduction and activation: the enzyme was ready to function upon reduction. Then, we turned to the substitution of the two most C-terminal cysteines, still individually or together. The resulting mutant proteins exhibited the same slow activation kinetics as the WT enzyme, but, while the WT enzyme was totally inactive without reduction, the mutants displayed a discrete activity with an increased K_m for their substrate, oxaloacetate. This activity could reach 20% of the activity of the fully activated enzyme, suggesting that the active site became at least partially functional. Combination of mutations at both ends, either triple (double N-terminal associated to one of the C-terminal cysteines), or quadruple, yielded fully active enzymes, insensitive to reducing treatments (Issakidis et al., 1994). These permanently active forms were strongly retained on affinity columns, requiring very high salt concentrations to be eluted, and, once purified, were rather unstable.

Meanwhile, another PhD student, Martine Lemaire, developed, under the supervision of Paulette Decottignies, a chemical derivatization approach targeting the putative active site histidine (Lemaire et al., 1994), and identified this active site histidine by sequencing the corresponding derivatized peptide. Later, she confirmed, using the same approach, that the active site of the enzyme comprised a His-Asp pair, like all the other malate dehydrogenases (Lemaire, Miginiac-Maslow, & Decottignies, 1996). This approach became very helpful for the confirmation of site-directed mutagenesis results. Indeed, it provided a simple means to test the accessibility of the active site of the enzyme. Thus, a pretreatment of the non-activated WT or N-terminal mutant enzymes with the derivatizing agent DEPC (which reacts with His) did not impair their subsequent activation by reduced Trx. In contrast, the pretreatment of the C-terminal mutants, or the activated WT enzyme prevented their subsequent activation by reduced Trx (Issakidis, Lemaire, Decottignies, Jacquot, & Miginiac-Maslow, 1996). Clearly, the active site was accessible in the C-terminal mutants and in all the activated enzymes but not in the N-terminal mutated and WT non-activated enzymes. This led us to propose that the formation of the C-terminal disulfide bridge shielded the access to the active site, and that the reduction of the N-terminal disulfide triggered a slow conformational change allowing the enzyme to reach an optimal activity conformation.

Started with PhD students from the Orsay University, specialized in Plant Biology, the NADP-MDH program continued with PhD students coming from other universities and specialized in biochemistry. They benefited from the know-how of Emmanuelle concerning site-directed mutagenesis. A hint about the nature of the conformational change of NADP-MDH upon reduction of its N-terminal disulfide bridge was brought about by Eric Ruelland, who obtained his master degree in Biochemistry at the "Ecole Normale Supérieure". Eric's PhD work led to propose a putative role of a conserved Cys situated in the middle of the primary sequence (Cys 207) in this conformational change. Indeed, a Trx-reducible disulfide bridge could be formed between the most N-terminal Cys (Cys24) and Cys 207 when the second N-terminal Cys (Cys29) was mutated. It is thus highly probable that an isomerization of the Cys24-Cys 29 disulfide into a Cys24-Cys207 disulfide occurs during the process of activation (Ruelland et al., 1997). Concerning the interaction of the C-terminal disulfide with the active site of the enzyme, a discussion with Kenth Johansson, a PhD student from the crystallography lab of Hans Eklund (Uppsala) attracted our attention to the fact that the two most C-terminal residues of the C-terminal extension were acidic residues (glutamate and valine) and that they could mimic the substrate oxaloacetate, a dicarboxylic acid. Thus, we deleted these two residues, or replaced them by neutral residues (e.g. glutamine for glutamate). We also combined these mutations with the elimination of one or both of the possible regulatory N-terminal disulfides (Cys24-Cys29 or Cys24-Cys207) by mutating the corresponding cysteines. Proteins

mutated at the most C-terminal residues had no activity in the oxidized form but were partially inhibited when pretreated with the histidine-specific reagent DEPC, showing that their active site was partially accessible. Proteins missing the N-terminal regulatory disulfides reached almost full activity without activation upon elimination of the negative charge of the penultimate Glu. These results strongly support a model where the C-terminal extension is docked into the active site through a negatively charged residue, acting as an internal inhibitor. They show also that the reduction of both possible N-terminal bridges is necessary to completely release the C-terminal extension from the active site (Ruelland, Johansson, Decottignies, Djukic, & Miginiac-Maslow, 1998). This interpretation was fully confirmed later, when the 3D structure of oxidized NADP-MDH was solved, in collaboration with the laboratory of Hans Eklund (Johansson et al., 1999). This took 7 years, and many purification campaigns of NADP-MDH. Indeed, it was very difficult to get a fully homogeneous preparation, the enzyme being prone to partial proteolysis at its N-terminal end, as verified by N-terminal sequencing. Finally, a bright and simple idea of Martine Lemaire, who spent a year as a post doc in the lab after her PhD, helped solve the problem: in the oxidized enzyme, the spontaneous proteolysis stopped after the deletion of the 15 most N-terminal amino acids. Thus, we prepared a recombinant MDH deprived of these 15 amino acids, a deletion that did not modify its regulatory properties, and obtained a very homogeneous preparation that yielded useful crystals. The structure showed that the N-terminal disulfide motif (Cys24-Cys29) was inserted between the two subunits of the dimer, thereby locking the domains of each subunit. The C-terminal extension was kept tight to the enzyme surface and shielded the access to the active site. Reduction of the N-terminal disulfide would loosen the interaction between the domains and give the enzyme the necessary flexibility. The reduction of the C-terminal disulfide would free the C-terminal residues from binding to the enzyme and make the active site accessible. As no structure of the reduced enzyme could be solved, this form being unstable, we turned to a collaboration with Jean-Marc Lancelin (University of Lyon) who was able to show, using two-dimensional NMR in solution, that in the reduced MDH, the C-terminal extension moves like a free peptide, allowing the direct sequence-specific assignment of its structure, while it is not visible in the oxidized enzyme, being probably stuck in the active site. Thus, it is obviously released from the active site upon reduction (Krimm, Goyer, Issakidis-Bourguet, Miginiac-Maslow, & Lancelin, 1999). The availability of the structure allowed to design useful mutations that led to the identification of active site arginines interacting with the acidic C-terminal residues, thereby preventing the access of the substrate, and of residues involved in cofactor specificity, unique to thioredoxin-dependent malate dehydrogenases (the permanently active forms are NAD-dependent), as well as of residues important for dimerization (Schepens et al., 2000; Schepens, Decottignies, Ruelland, Johansson, & Miginiac-Maslow, 2000; Schepens, Ruelland, Miginiac-Maslow, Le Maréchal, & Decottignies, 2000). The PhD work of Isabelle Schepens, after her master degree in structural biology, showed also that the specificity for NADPH could be changed for NAD by site-directed replacement of selected residues, without changing the thioredoxin dependency, and that the inhibition of activation by the oxidized form of the cofactor (first observed by Scheibe & Jacquot, 1983) was retained in the NAD-specific mutants, suggesting that the cofactor interacted, through its positive charge, with the negatively charged C-terminal residues, preventing the release of the C-terminal extension from the active site.

An attempt to confer redox regulation on the thermostable NAD-dependent MDH from *Thermus flavus* by grafting the extensions of sorghum NADP-MDH showed that a successful transfer of redox regulation properties requires the grafting of both extensions, but also the introduction of specific hydrophobic residues in the core part of the protein. These residues are very likely involved in the interaction between monomers, and structural changes at the active site (Issakidis-Bourguet, Lavergne, Trivelli, Decottignies, & Miginiac-Maslow, 2006). The MDH work was completed by the demonstration, by Stéphane Lemaire, who came back to Orsay as a permanent CNRS researcher, after a post-doctoral stay in Geneva, that the complex activation mechanism of this enzyme was specific to higher plants. In the green alga *Chlamydomonas* only the C-terminal disulfide is present and full activation is reached within seconds (Lemaire et al., 2005). Then, obviously the activation mechanism evolved toward higher complexity when the role of NADP-MDH in exporting reducing equivalents (the so-called “malate valve”: Scheibe, 2004) became more prominent.

The sequential model of activation of NADP-MDH was also supported by the determination of the redox potentials of the different disulfide bridges of NADP-MDH (Hirasawa et al., 2000), in collaboration with the late David Knaff. The N-terminal disulfide was shown to be less negative, i.e. more easily reducible than the C-terminal disulfide. The latter was more negative than thioredoxins, hence required an excess of reducing power to be reduced. It can thus be hypothesized that depending on the ambient reducing state of the chloroplast, NADP-MDH might exist in a partially reduced form which would then be prone to faster activation when sufficient reducing power becomes more available.

The occurrence of the Cys24-Cys 207 disulfide raised the problem of the accessibility to thioredoxin of Cys 207, located in the middle of the primary sequence. The problem was tackled in the PhD work of Aymeric Goyer, holding a master degree in chemistry and biochemistry from the University of Rennes, who studied the interaction of Trx with NADP-MDH through the creation of mixed disulfides between a monocysteine mutant of Trx and specific cysteines of its target enzyme (Goyer et al., 1999; Goyer, Decottignies, Issakidis-Bourguet, & Miginiac-Maslow, 2001). Indeed, inspired by the pioneering work of Fred Hartmann (Brandes, Larimer, & Hartman, 1996), this approach allowed trapping the cysteines targeted by the reductant. Aymeric was able to show that Cys 207 was the only, among the four internal Cys, that could form a mixed-disulfide-bound complex with monocysteine Trx, hence was accessible to Trx. Having established efficient protocols for generation of mixed disulfides, Aymeric applied this technology to set up a monocysteine Trx affinity column for trapping putative Trx targets. This allowed us to develop proteomic studies aimed at identifying Trx targets at a whole cell level, with the mass spectrometry facilities developed by Paulette Decottignies and Pierre le Maréchal at the Orsay Institute of Biochemistry (Goyer et al., 2002; Lemaire et al., 2004; Marchand et al., 2004, 2010). This now widely used technology allowed the identification of a large number of new Trx targets. This global proteomic approach was further developed by Stéphane Lemaire to study redox-dependent post-translational modifications of proteins, such as glutathionylation (Chardonnet et al., 2015; Michelet et al., 2005; Zaffagnini et al., 2012) or nitrosylation (Morisse, Zaffagnini, Gao, Lemaire, & Marchand, 2014).

The early work on NADP-MDH activation, started in Nancy, had revealed the existence of two different thioredoxins in chloroplasts: Trx m and Trx f, the former seeming to activate specifically NADP-MDH (Jacquot et al., 1976, 1978). However, at the turn of last century, the Arabidopsis whole genome sequencing revealed that the plastidial Trxs of this organism comprised 10 isoforms: 4 *m* type, 2 *f* type, 1 *x*, 2 *y* and 1 *z*. This feature prompted us to reinvestigate the specificity of these various isoforms in a more physiological context. First, we tested, *in vivo*, the ability of these isoforms to compensate for TRX deficiency of a *Saccharomyces cerevisiae* mutant strain, in collaboration with Yves Meyer (University of Perpignan). Seven Arabidopsis plastidial Trx (four *m*, two *f* and a newly discovered TRX *x*) were expressed in a *Trx*-deficient strain of yeast in a putative mature form. None of these heterologous *Trx* was able to restore growth on sulfate or methionine sulfoxide of the mutant cells. When we tested their ability to rescue the oxidant-hypersensitive phenotype of this strain, we found that Trx *m* and Trx *x*, but not Trx *f*, modified the tolerance to oxidative stress induced by either hydrogen peroxide or an alkyl hydroperoxide. Arabidopsis Trxs *m*1, *m*2, *m*4 and *x* induced hydrogen peroxide tolerance, like the endogenous yeast thioredoxins. Trx *m*3, in contrast, rendered yeast more susceptible (Issakidis-Bourguet, Mouaheb, Meyer, & Miginiac-Maslow, 2001). In parallel, we produced all the plastidial Trx as recombinant proteins and checked their specificity *in vitro*, toward known targets, or putative targets involved in oxidative stress resistance. This research was led essentially by Emmanuelle Issakidis-Bourguet, who supervised the PhD work of Valérie Collin. The results confirmed the specificity of the 2 Trx *f* for FBPase. MDH appeared to be activatable by Trx *m*1, 2 and 4, but also by Trx *f* that seemed to be even more efficient than Trx *m*. In contrast, neither Trx *x*, nor Trx *y* were able to activate these two enzymes, but were efficient in donating their reducing equivalents to 2Cys-Prx (for Trx *x*) and Prx Q (for Trx *y*), two thioredoxin-dependent plastidial peroxidases (Collin et al., 2003, 2004; Lamkemeyer et al., 2006). These new thioredoxins had also a less negative redox potential. This was the first direct evidence for the role of specific chloroplastic Trx as electron donors to thiol-dependent peroxidases. This finding opened the way to a new research program, further developed by Emmanuelle Issakidis-Bourguet, and aimed at deciphering the role of Trx in oxidative stress responses *in vivo* in Arabidopsis, through the use of specific knockout mutant plants now freely available in libraries (Keryer, Collin, Lavergne, Lemaire, & Issakidis-Bourguet, 2004; Laugier et al., 2013; Rahantaniaina et al., 2017; Thormählen et al., 2013, 2017; Vanacker, Guichard, Bohrer, & Issakidis-Bourguet, 2018, among others).

Thus, the redox program first launched by Pierre Gadal in mid-70s developed considerably during the following decades, integrating a net of fruitful national and international collaborations. Without all these collaborations, we would have never been able to solve the complex mechanism of activation of NADP-MDH, the first example in the field of redox regulation of the so-called “intrasteric inhibition,” a concept developed by Bruce Kemp for protein kinases (Kobe & Kemp, 1999). In the lab, this very active and enthusiastic research work was done in a friendly and relaxed atmosphere, with many interactions with the other members of the lab, from the morning coffee prepared by a devoted Hungarian lady, Margareth Nagy, and attended by everybody, including Pierre Gadal, to birthday celebrations with a home-made cake and champagne. Sometimes we also enjoyed excellent cakes coming from the local bakery that Bob Buchanan brought to the lab each time he was visiting us.

This research program allowed also most of our PhD students to get permanent positions either in French institutions (CNRS for Michel Droux, Emmanuelle Issakidis-Bourguet, Eric Ruelland and Stéphane Lemaire, INRA for Frederic de Lamotte-Guéry and Martine Lemaire) or abroad (a biotechnology company in Switzerland for Isabelle Schepens, Oregon University, USA, for Aymeric Goyer). Two of them (Emmanuelle and Stéphane) are still active in the redox domain and continue to develop our knowledge about the fascinating topic of redox regulation.

Pierre Gadad officially retired in 2003, but was regularly present at the Institute during several following years, as an emeritus. During these years he acted as an advisor, following the research progress of PhD students belonging to the Orsay Doctoral School of Plant Sciences and helping them find interesting post-doctoral stays. His advice was highly appreciated.

2.3.2 Michel Droux recollections from 1980 to 1988

My first memories of Professor Pierre Gadad go back to the years 1980–1981, when I was taking his courses in plant physiology at the University of Paris XI in Orsay. Exciting classes where he told us stories about the life of the plant. Always smiling, and in constant dialog with his students. At that time, I was finishing my studies (now M1) for a master's degree in Ecology of organisms and populations. In line with this aspect, I continued my studies within the framework of a DEA (now M2) in the doctoral school of Professor Miginiac (University of Paris VI), with for training course a purely biochemical subject presented by Professor Pierre Gadad. I thus followed his teaching in the field of thioredoxins (TRX) under the supervision of the future Doctor Jean-Pierre Jacquot and in collaboration with Myroslawa Miginiac-Maslow. I must confess that I had no notion of protein biochemistry and that I learned everything at the bench with the encouragement of Pierre Gadad, a human and respectful person. On the other hand, my two supervisors had to tear their hair out and had headaches. What a bad experience for Jean-Pierre, I was his first PhD student.

This scientific project focused on the study of the enzymatic mechanisms involved in the photoregulation of chloroplastic enzymes, the Ferredoxin-ThioRedoXin (FdTRX) system. My aim was to purify and characterize the ferredoxin-thioredoxin reductase (FTR). This approach was then pursued during a post-doctoral internship in the laboratory of Professor Bob B. Buchanan at the University of California, Berkeley that I cannot dissociate from my Pierre Gadad experience.

In photosynthetic organisms, light plays an essential role in the regulation of metabolism (Buchanan, 1980). This role is reflected by post-translational modifications affecting the redox state of several target enzymes, which are inactive in the dark and become active in the light. At the beginning of this work, several hypotheses were proposed to account for these photoregulatory phenomena which are dependent on photosynthetic activity and which are mimicked in the dark by the addition of a chemical reducing agent, dithiothreitol (DTT) (Jacquot et al., 1976). The three proposed systems used either redox components associated with the thylakoid membranes of the chloroplast (the Light Effect Mediator (LEM) system) (Anderson, Nehrlich, & Champigny, 1978) or located in the stroma (the Ferredoxin-ThioRedoXin (FdTRX) and the Ferraltherin systems) (Lara, De la Torre, & Buchanan, 1980; Wolosiuk, Buchanan, & Crawford, 1977). My thesis and post-doctoral work demonstrated that the ferredoxin-thioredoxin system is the unique physiological system that efficiently couples light energy to the photoactivation of several chloroplastic target enzymes. This system is composed of ferredoxin (Fd), ferredoxin-thioredoxin reductase (FTR) and thioredoxins (TRX m and f). At that time, thioredoxins were classified into two functional categories characterized by their target specificity, thioredoxin m associated with the photomodulation of NADP-malate dehydrogenase (NADP-MDH) and thioredoxin f modulating the activity of four of the Calvin cycle enzymes (including fructose-1,6-bisphosphatase, FBPase) (Droux, Crawford, & Buchanan, 1987; Droux, Jacquot, et al., 1987).

The study of the FdTRX system was made possible by the development of a rapid assay method for ferredoxin-thioredoxin reductase (FTR) activity. This method, called the reconstituted chloroplastic system, requires thylakoids (or electron transporters), purified protein components (Fd, FTR, TRX m or f) and a target enzyme (NADP-MDH or FBPase) (Jacquot, Droux, et al., 1984). The determination of the activity of FTR was performed by measuring its ability to promote activation of the target enzyme via TRX. The degree of activation of the target enzyme is finally assessed by measuring its catalytic activity.

The implementation of this system has led to a fine characterization of the FTR of plants of C3 type (spinach), C4 type (corn), and a cyanobacterium (*Nostoc muscorum*) (Droux, Jacquot, et al., 1987). At this stage, in the absence of

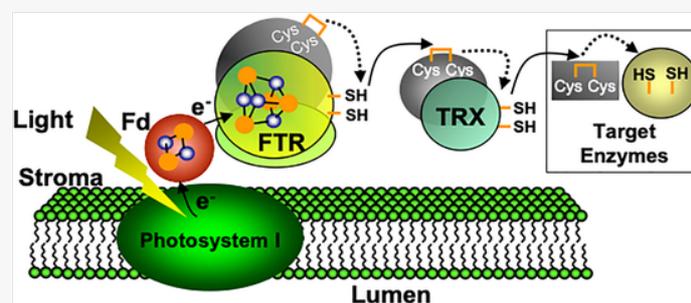
molecular biology tools, FTR characterization used conventional protein purification techniques from kilograms of plants leaves (e.g. during my thesis I may have ground more than 100 k of spinach leaves). FTR is a protein that is poorly represented in the chloroplast and rather unstable. Therefore, a major advance in its purification has been achieved using affinity chromatography. This step, based on the coupling of ferredoxin (Fd) to a solid support (Shin & Oshino, 1978), allowed, in addition to the purification of FTR, the isolation of several chloroplastic proteins using ferredoxin as an electron donor for their activity (e.g. ferredoxin-NADP reductase, nitrite reductase, glutamate synthase, and sulfite reductase) (Knaff & Hirasawa, 1991). This method has thus led to the concept of the protein-protein complex (Fd-FTR) within the FdTRX system. These protein-protein interactions involve negative charges located within a domain of Fd and positive charges at the FTR level (Hirasawa et al., 1988).

FTR is a heterodimeric protein, consisting of two different polypeptides, and carrying a 4 Iron-4 Sulfur center. A comparison of the masses of the polypeptides of the protein in different species revealed the presence of one conserved polypeptide in mass and another of variable mass (Droux, Jacquot, et al., 1987). From pure proteins we prepared antibodies in rabbits. One special memory was working with Pierre Gadal on Sundays taking care of the rabbits, our antibody factories. Yes, we had to clean the cages, give them food. So there, my surprise was to see the University professor turn into a farmer wearing wooden clogs or sabots as they are known in France. No matter, we had to get down to work, always with the same joviality. My “anti-FTR” rabbits responded well, because antibodies were the basis for the notion of similar and variable subunits among three organisms analyzed (Droux, Jacquot, et al., 1987).

Furthermore, analysis of the cysteine content of these polypeptides indicated the presence of a disulfide bridge within the conserved polypeptide. This important result introduced the concept of an active subunit in the transfer of the reducing power from photoreduced Fd to TRX, through thiol-disulfide exchanges. Following the physicochemical characterization of FTR, the project consisted in elucidating, using biochemical approaches, the photoactivation mechanism of NADP-MDH and FBPase. These approaches, developed in the reconstituted chloroplast system (Droux, Miginiac-Maslow, et al., 1987), were complemented by a study in the intact chloroplast. For this purpose, a specific radioactive probe (iodoacetamide) of the SH groups was first used, but a great progress was made thanks to the introduction of thiolite (or monobromobimane) (Crawford, Droux, Kosower, & Buchanan, 1989). This probe has the advantage of rapidly crossing cell membranes and allowing direct measurement, by fluorescence, of the redox status of FdTRX system proteins isolated from intact chloroplasts by immunoprecipitation. These experiments showed the reduction of the disulfide bridge of the conserved FTR subunit, of the canonical site of TRX, and of disulfide bridges on the target enzymes during incubation in the reconstituted chloroplast system, or of the intact chloroplasts, in light, thus clarifying the concept of photoactivation of chloroplastic enzymes (Crawford et al., 1989) (Fig. 3).

alt-text: Fig. 3

Fig. 3



Schematic representation of photoactivation of target enzymes in chloroplasts. Electron transfer from the photoreduced ferredoxin to the Fe-S center of FTR achieves light activation. FTR thus converts the electron flow into a thiol signal, which, through a thiol-disulfide exchange, reduces the active site of the thioredoxins (Dai et al., 2000; Walters et al., 2005). The thioredoxins, by a thiol-disulfide exchange, will then reduce the regulatory site of the target enzymes. This oxidation-reduction cascade of disulfide bridges leads to the transformation of an inactive target enzyme present in the dark into an active target enzyme in the light.

Beyond science, Pierre Gadal helped me a lot during my thesis, and even beyond since he visited me in Berkeley and then we attended a Gordon conference together. He introduced me there to another University Professor, Roland Douce and his colleagues, a move that proved determinant for obtaining my future CNRS position and thus the rest of my career.

2.3.3 Emmanuelle Issakidis-Bourguet: Recollections from 1986 to 2003 and beyond

I met Pierre Gadal in 1986 as my Professor in Plant Physiology at Orsay University (Paris-Sud 11). I don't have a clear memory of his lectures, but for sure they confirmed my choice to study biology at the molecular level. I have been attracted to plants since my childhood, and studying plant molecular functions has proven to be really exciting to me. I also don't remember exactly how I managed to get a 6-week laboratory internship in Prof. Gadal's lab, but what is certain is that it determined the outcome of my studies and my entire subsequent scientific career.

During this internship, mentored by Pierre Gadal, I was supervised by his PhD student Ridong Chen. This was a very nice scientific and human experience. I loved the experimental action and the intellectual activity which came with it. The subject of the internship was to measure and compare NADP-dependent isocitrate dehydrogenase activities and immuno-reactivities with an antibody obtained by Ridong, from different biological materials. This work involved not only plant extracts but also extracts from bacteria and animal organs. I remember Ridong arriving at the lab in the morning with bloody animal samples in his ice box, which he had retrieved from colleagues in the neighboring Animal Physiology laboratory. It was quite difficult for me to start the working day with this type of material. I think that this practical experience definitely convinced me that I would be working on plants and it made me want to make a career out of it later on.

We, with Ridong and Claire, another undergraduate student I was paired with, did a job good enough allowing our data **Q13** to be published 2 years later (Chen, Bismuth, Champigny, & Gadal, 1989; Chen et al., 1989). How proud I was of this very first personal contribution to science! I wanted many more to come afterwards... As I mentioned, this first internship was also a very positive experience on a human level. I immediately liked the working atmosphere of a research laboratory. Its international dimension, Ridong was Chinese, the visits of collaborators from abroad. I really liked this multicultural context. In Pierre Gadal's lab, there was a very good working atmosphere, lots of exchanges over coffee, celebrating personal events (birthdays, baby births and weddings...). Prof. Gadal was attentive to everyone, always kind and caring with all lab members.

I very much wanted to come back to work in his laboratory, but in order to be sure about my career choice for the future, for the next 2 years I pursued my studies at Orsay University and did voluntary internships in laboratories where the research was more applied (at INRA and in a private company), to finally confirm my interest for fundamental research.

I chose specializing in biochemistry and plant physiology and once more had Prof. Gadal as teacher (course on plant metabolic pathways). Logically, in 1989, I applied for the DEA (Diplôme d'Etudes Approfondies) which, I think, P. Gadal had created and co-directed with Prof. Francis Quétier. This training course was the equivalent of a Master's degree specialized in *Plant Molecular and Cellular Biology* and was ending with a 6-month full-time internship. My plan was to be back working in P. Gadal's lab on the isocitrate dehydrogenase project. But it happened that P. Gadal proposed an internship to one of my classmates who came from a collaborating Spanish team and he reassured me that the research project he directed could give rise to two internship subjects. I remember that Francis Quétier, concerned about the continuation of my Master's degree and the possibility of pursuing a PhD thesis, strongly advised me to consider another internship subject proposed in the same laboratory. I followed his advice and this is how I started working on NADP-dependent malate dehydrogenase (NADP-MDH), initially under the direction of Jean-Pierre Jacquot and Claude Créatin. Looking back, this change of plan, which at the time had frustrated me, was a great opportunity. Claude had isolated the cDNA of NADP-MDH from sorghum and Jean-Pierre cloned it in a vector for expression in *E. coli*. My mission was to increase the production rate of the recombinant enzyme in order to facilitate its purification and obtain it in quantities large enough to conduct the structure-function study of its regulatory mechanism by a site-directed mutagenesis approach. This was done and it paved the way for my thesis project...

At that time, obtaining a site-specific modified protein was a long-lasting and hard task. PCR was not readily available, synthetic DNA production was just starting and not commercially available. Notably, automated DNA sequencing was not accessible yet. Following the method described by Kunkel in 1985, to introduce a point mutation in a cDNA, it was necessary, first, to obtain a single stranded DNA version using bacteriophage technology, second, to perform its in vitro copy using a mutagenic oligonucleotide (primer extension), third, to transfer it into a bacterial strain where the uracil-containing parental DNA strand (bearing the starting wild-type sequence) would be counter-selected. All this process had a limited efficiency (50–60% in my hands) and the suspense about obtaining a mutagenized DNA lasted until

getting DNA sequencing results. Sequencing was another fastidious task in the process since it was manually operated. We used to perform Sanger sequencing with ^{35S} labeled ATP and handling the thin layer acrylamide gels was always a risky step. A mutant clone bearing the desired modification once identified, the cDNA (or possibly a part of it when a judicious sub-cloning could be done) had to be entirely sequence verified prior to transfer into an expression vector for recombinant protein production. Nowadays, mutagenesis is performed using PCR and sequencing is custom realized; chemical synthesis of a site-specific modified DNA sequence encoding a whole protein has even become financially affordable and the development of the CRISPR/Cas9 technology now allows mutagenesis to be performed in vivo. In the early 90s, at the time of my PhD work, tagged protein technology to simplify purification by affinity chromatography had only just been developed and had not yet been disseminated. Purifying a mutated recombinant protein could be another fastidious task. For NADP-MDH, the purification procedure needed a number of days of experimental work including several ammonium sulfate fractionations and chromatography steps. One can then imagine the satisfaction of obtaining a purified mutated protein, when the most interesting part of the work could finally start...

The NADP-MDH project in P. Gadal's lab has been a real saga, i.e. by definition a long story, with a series of incidents and sort of heroic achievements. Myroslawa Miginiac-Maslow has described the whole project to which I had the chance to contribute. NADP-MDH is reductively activated in the light by the ferredoxin/thioredoxin system and it has the particularity, among thioredoxin-dependent enzymes, to be totally inactive in its oxidized state. Previous work from Paulette Decottignies and Myroslawa had revealed that the two cysteine residues located near the N-terminus of the enzyme were reduced upon its light activation, strongly suggesting that they could be linked in a disulfide bridge. My PhD thesis work, under the supervision of Myroslawa, started with the production of mutant proteins where these putative regulatory cysteines were substituted, separately or simultaneously. Their replacement gave enzymes that still needed a reducing treatment to be active (incident!) but we noticed that their activation kinetic was much accelerated in comparison with the wild-type enzyme. Then, I created new mutated proteins where the two conserved cysteines located in the C-terminal part of NADP-MDH, corresponding to a sequence extension specific to the redox regulated MDH isoforms, were substituted. These proteins still needed a reducing treatment to be fully active and their activation was as slow as the wild-type enzyme, but they showed a substantial activity level (ca. 20% of the maximum activity) in the oxidized state, strongly suggesting that they could be involved in the activation process, possibly in a second regulatory disulfide (Issakidis et al., 1992). Then, by combining the mutations of both the N- and C-terminal cysteines, we could obtain a thioredoxin-independent fully active NADP-MDH (achievement!) (Issakidis et al., 1994; Issakidis, Decottignies, & Miginiac-Maslow, 1993).

Then, in 1994 I went to Australia for a post-doctoral period in J. Andrews' laboratory (ANU Canberra) when the new building 630 on the Orsay university campus hosting IBP—Institut de Biotechnologie des Plantes—founded by P. Gadal and F. Quétier was completed. When I came back, I was able to get a CNRS permanent position in the team directed by Myroslawa with a research project on redox regulation by thioredoxins, including my favorite target enzyme. Since my PhD work, we had the ambition to transfer the redox control properties of NADP-MDH to a thermostable MDH isoform. To do so, I had visited Prof. T. Beppu's laboratory in 1992, at the occasion of my trip to Japan attending the IXth International Congress on Photosynthesis, which provided us with the cDNA clone for NADP-MDH from the thermophilic bacteria *Thermus flavus*. Thanks to the accumulation of data obtained over a period of almost 15 years by me and the other subsequent PhD students supervised by Myroslawa or Paulette, and the valuable help from our collaborators, experts in structural biology, we were able to reach our goal (achievement!). I personally regret that this accomplishment did not receive the interest that, in my opinion, it should have had. The corresponding paper was published in 2006 (Issakidis-Bourguet et al., 2006), after several attempts at publication in biochemistry-oriented journals where one of the reviewers even pointed out that he/she could not see the interest of our work. Pierre Gadal certainly was not one of those “pitbull reviewers.” Although he could not be directly involved in all the research projects underway in his laboratory, he always kept updated about everyone's progress. His criticism was always constructive. He used to sow the seeds of doubt and reflection in your mind and would come back to you later to check whether they had germinated...

At the beginning of my PhD work, obtaining purified recombinant NADP-MDH protein in large quantities allowed us to produce antibodies. We had them made by rabbits raised at the animal house of the laboratory of Animal Physiology of the Orsay campus. Jean Vidal helped me for the injections of the rabbits, and the final step of their bleeding, which was quite disturbing, not only for the rabbits... They used to show resistance. I still have a scar on my forearm in

memory of the “MDH rabbit.” No one wanted to finish off the rabbits. It was said that P. Gadal was in charge of it and that the rabbits would eventually end up on his plate... If that were true, I do not think it showed his lack of sensitivity, but rather a reference to his rural origins.

Over recent years until 2017, I met him from time to time at IPS2. The Institute of Plant Sciences Paris-Saclay, housed at building 630, was created in 2015 from the combination of 3 plant research institutes—including IBP—located in the southern Paris region. P. Gadal was passing by to give and enquire as to the latest news, with his cans for softened water for his fish tanks.

In retrospect, I realize that Pierre Gadal was present at all the important steps of my student (first internship, Master, PhD: he was President of my defence jury) and professional (CNRS position) lives, and I'll always be most grateful to him for all the support and opportunities he gave me. I will warmly remember Professor Gadal as a good person and a great scientist.

2.3.4 Martine Lemaire-Chamley: Recollections from the Orsay era (1991–1997)

Was it a long time ago? Yes, it was in another century, between 1991 and 1997, when I discovered Research and Plant Biotechnologies in Orsay. At that time, plants and enzymes were my focal points. Thus in 1991, I performed my first 8-week training in the Plant Physiology laboratory headed by Pr. Pierre Gadal. I started my research activity in a cold room to purify plant enzymes and then in front of the spectrophotometer to measure their enzymatic activity, under the supervision of Paulette Decottignies. Of course, after this very interesting and successful internship, I wished to dedicate myself to scientific research and therefore I applied for the “DEA in Plant Molecular and Cellular Biology” directed by Prs Pierre Gadal and Francis Quétier and was very happy to be selected. Plant enzymology was not that much fashionable at that time, when molecular biology and bioinformatics were starting to take-off in the plant field. However, I managed to obtain a PhD grant to continue my work, still on the Orsay campus, first in the “valley” in building 430, then at the Institut de Biotechnologie des Plantes on the Saclay plateau, an institute that Pierre Gadal initiated and co-supervised the construction of. There, we benefited from unbelievable working conditions, with so much space and functionality. For a PhD student, Pierre Gadal was a very impressive person because of his responsibilities, of course: he was at the top of our hierarchical pyramid and at the interface with the rest of the scientific or administrative world... Tall and rapid in his movements, his speciality was to appear suddenly in the corridor to ask for someone or something. He scared me a couple of times, at night in the lab, when I emerged from the cold room where I was watching my chromatography column and almost bumped into him. He made me nervous by searching frantically for someone to carry important files to the ministry the day of the deadline. But he was also a very caring person, very concerned by his staff: he moved me to tears during my thesis defense, evoking a colleague who passed away way too early. I was proud that he trusted me enough to offer me a short-term teaching position as an assistant professor (ATER) after my post doc in Germany, before I applied successfully for a permanent position at the INRA. In brief, through his goodwill to everyone and his investment in research and teaching management, Pierre Gadal, together with the permanent researchers present in the lab at that time, showed me how a scientific lab could be run properly, in a fully serene working environment. I am very grateful to him for his outburst of good vibes during the first years of my scientific career.

2.3.5 Aymeric Goyer's recollections of the Orsay era (1998–2001)

When I think about persons who have made a difference in my life, Pierre Gadal comes immediately to my mind. I did my DEA (eq. MSc) and PhD research theses at the Institut de Biotechnologies des Plantes that Pierre Gadal founded, in the group directed by Myroslawa Miginiac-Maslow, between 1998 and 2001. My first encounter with Pierre Gadal was during my interview in the spring of 1997 to get into graduate school in the Plant Molecular and Cellular Biology program at the University of Paris XI. I was finishing my undergraduate studies at the University of Rennes 1 in Bretagne (Brittany), and I had applied to several graduate schools throughout France. The Plant Molecular and Cellular Biology program was my first choice because of the plethora of top plant science laboratories involved, including Pierre Gadal's lab. The interview was a short 10–15 min roundtable with the members of the selection committee. I had never met any of those members before, but each had a nametag in front of them. When members would ask questions, I would take a pick at their nametags, and I quickly realized that many of the top plant scientists from the region were gathered right there in front of me. I still remember Pierre Gadal's question about my internship at Bretagne Biotechnologies Végétales, a question that was indirectly related to Brittany of course, a region where we both are

originally from (Breizh da viken!!!). Soon after, I would learn that I had been accepted in the Plant Molecular and Cellular Biology program. So, I moved to the beautiful woody Orsay campus and started to take courses and to rotate between labs at several scientific centers throughout the Paris region, to eventually settle down in the Redox Regulation group at the Institut de Biotechnologies des Plantes where I would do my thesis research for the next 4 years. To me, this lab offered the right balance of biochemistry, molecular biology, and plant physiology that I was looking for, and the people (faculty, technicians, students) made me feel that it was the right environment for me to go through the stressful years of graduate school. There, I studied the regulation of the activity of NADP-malate dehydrogenase, a mechanism that involves molecular interactions of the enzyme with thioredoxins via the formation of specific cysteine disulfide bridges. I made my first steps into molecular biology by creating mutations in proteins coding sequences by site-directed-mutagenesis, and by verifying the mutations in the plasmid sequences by running long polyacrylamide gels, a tedious protocol that would astonish the current generation of graduate students. I would then produce the corresponding recombinant proteins in *E. coli* and purify the enzymes by using a series of size exclusion, ion exchange, and affinity chromatography columns. Once purified, I would prepare mixtures of mutated thioredoxins and NADP-malate dehydrogenase, and analyze the resulting complexes by SDS-PAGE and HPLC. The lab had become expert in all these techniques, capable of obtaining several milligrams of highly purified proteins. So, my first experiences at the bench went without major roadblocks, and in just a few months, I was lucky enough to have my research results published (I would learn later on that research is often made of seldom ups and long excruciating downs). During these years, I had the chance to get to know Pierre Gadal better. Although he was quite intimidating at first, he was somewhat the opposite of the first impression he projected, someone very approachable and always happy to converse, whether it be in the lab (unaware of how unsettling interrupting a graduate student running an experiment can be), in the hallways of the institute, or at the coffee breaks. During one of those conversations, after returning from the Plant Biology 2000 annual meeting in San Diego, CA, where he received the Corresponding Membership Award, he informed me of an open post-doctoral position at the University of Florida in the laboratory of Andrew Hanson whom he had met there, and encouraged me to apply. Which I did. I then moved to Florida in January 2002 shortly after receiving my PhD degree, a decision that drastically changed the course of my professional and personal life.

3 Nitrogen metabolism pathways and regulation

3.1 Akira Suzuki's recollections

Pierre Gadal, Professor at the University of Nancy I and later Paris XI, founded a laboratory of international reputation in plant science. I have been privileged to have worked with him in this laboratory for 3 years at the Faculty of Sciences, University of Nancy I (presently University of Lorraine) in 1978–1981 and then for 3 years at the Faculty of Sciences, University of Paris XI in 1981–1984. As initial studies are determinant for the subsequent development of research, my recollections here describe what happened in the first years-work in this lab. Besides, a review article is **Q14** dedicated to Pierre's memory (see Chapter 4). The review refers to the study of the primary nitrogen metabolism in his lab in the 1970s and the early 1980s together with the outcomes of worldwide researchers working in the field of primary nitrogen metabolism.

3.1.1 Early years in Nancy

It all started by an appointment in the office of Prof Pierre Gadal in the Faculty of Sciences, University of Nancy I. After the interview, I came out of his office located at the end of fifth floor of the circular building, holding in my hands two recent master theses on nitrogen metabolism defended by two students in his lab. This happened in 1978. The study was initiated in the Plant Biology Laboratory (ERA au CNRS 799) as a DEA (today Master) student after receiving a Bachelor of Science degree in plant biology at the Faculty of Science, Tohoku University, Japan. My first laboratory notebooks delineate the daily notices of basic protocols and more advanced methods on nitrogen metabolism.

Year 1978:

- September 11: Seeding cultures of five plants (maize, rice, bean, pea and barley) on Petri dishes using a medium of **Q15** Hoagland and Arnon (1938) or Lockard (1959).
- September 27: Crude enzyme extraction and assays of nitrate reductase (NR), nitrite reductase (NiR), glutamate dehydrogenase (GDH), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate-

oxaloacetate transaminase (GOT), and organelle markers of triose-phosphate isomerase, fumarase and catalase.

- September 27: Spectroscopic determination of protein concentrations (Scopes, 1974).
- October 11: Organelle/cytosol localization of the enzymes by differential centrifugation.
- October 13: Biuret-Folin colorimetric assay of soluble proteins (Lowry, Rosenbrough, Farr, & Randall, 1951).
- October 26: Organelle localization of the enzymes by ultracentrifugation on linear or discontinuous density sucrose gradients.
- November 23: Memo “Could you prepare 20 Petri dishes and place maize seeds today? This will allow me to do experiments on roots on Saturday.” Pierre Gadad sometimes tried to do experiments himself despite his numerous national and international commitments.
- November 28: Appending asparagine synthetase (AS) and carbamoylphosphate synthase (CPSase) to the localization study.

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Year 1979:

- March 13: Assay of Fd-GOGAT by separating glutamate (Glu) from glutamine (Gln) by paper chromatography for 4 h (phenol/H₂O: 4/1, v/v) and measuring Glu and Gln by ninhydrin.
- March 19: Profiling of activity of NiR, Fd-GOGAT and NADH-GOGAT in greening etiolated leaves for 30 min, 1 h, 2 h up to 72 h.
- April 23: Spectrophotometric GOGAT assay by measuring reduced MV oxidation under anaerobic conditions (Asada, Tamura, & Bandurski, 1969).
- May 14: Profiling of activity of NiR and Fd-GOGAT using cycloheximide to inhibit protein synthesis on cytosolic ribosomes in greening etiolated leaves.
- May 28: Profiling of activity of NiR and Fd-GOGAT using chloramphenicol to inhibit protein synthesis on plastidial ribosomes in greening etiolated leaves.

During all this time, the lab members had the advantage of interacting with visiting scientists from France and abroad. Since the 1970s, Pierre Gadad worked with leader scientists from the UK and Canada in the field of nitrogen metabolism. He and the CNRS researchers of the lab made available a range of analytical tools, skills and competence in the study of isoenzymes of nitrogen assimilation: isolation and measurement of inorganic and organic molecules and proteins, chromatography by gel filtration, ion exchange and hydroxylapatite, electrofocalization, PAGE, etc., used for the PhD projects. In a series of experiments, we reported the localization of the key enzymes in the plastids, mitochondria, peroxisomes and cytosol of the non-photosynthetic roots of five plants (Suzuki, Gadad, & Oaks, 1981).

Meanwhile, Pierre Gadad launched a new approach of immunocytochemical localization of the enzymes implicated in the carbon metabolism, one of the two axes of the laboratory. Magnificent and specific images of the indirect immunofluorescent localization of the enzymes of CO₂ fixation by a lab mate unequivocally clarified the carbon metabolic networks in the leaf. The lab members shared this new concept and technology, and each of us carried out the purification of proteins of interest to homogeneity, immunization of rabbits by initial and booster antigen injections, blood collection and IgG isolation, etc. Years later, immunochemistry became one of the indispensable tools of plant biology to study target proteins, e.g., in screening of recombinant DNA libraries and DNA cloning, immunohistochemistry, quantification of specific proteins, identification of cross-reacting materials including recombinant proteins, immunotitration of proteins such as in vivo synthesized proteins marked with labeled amino acids, etc.

3.1.2 Later in Orsay

Following the appointment of Pierre Gadad as Professor in Orsay in 1981, most of the lab members moved with him to the Plant Molecular Physiology Laboratory (ERA au CNRS 799) at the Faculty of Sciences, University of Paris XI.

After installation in Building 430, he organized a series of visits: Institute of Nuclear Physics (Building 100) and Linear Accelerator Laboratory (Building 200) located on the campus along the Yvette river, INRA Versailles (present INRAE), etc. In the subsequent years, the INRA Versailles center has become the site of my research activities after I became an INRA researcher.

The study in Orsay aimed at answering the biological questions about GOGAT and primary nitrogen metabolism.

- Since the discovery of Fd-GOGAT in pea leaves in 1974 (Lea & Mifflin, 1974), the coexistence of Fd-GOGAT and NAD(P)H-GOGAT activities was reported in non-photosynthetic roots (Mifflin & Lea, 1975). We provided evidence that Fd-GOGAT and NADH-GOGAT were distinct proteins encoded by separate genes in a variety of plant species (Suzuki, Jacquot, & Gadal, 1983; Suzuki, Vidal, & Gadal, 1982; Suzuki, Vidal, Nguyen, & Gadal, 1984).
- The structure and kinetic properties of the major Fd-GOGAT in the green leaves were characterized using the purified Fd-GOGAT protein (Suzuki & Gadal, 1982).
- The systemic in silico analysis reviewed the studies of structure, function and regulation of Fd-GOGAT and NAD(P)H-GOGAT in animals, archaea, bacteria, yeasts, cyanobacteria, algae and higher plants (Suzuki & Gadal, 1984).
- Other questions remained unanswered. Since the discovery of Fd-NiR activity in barley roots in 1966 (Joy & Hageman, 1966) and Fd-GOGAT activity in pea roots (Mifflin & Lea, 1975), the physiological function of these Fd-dependent enzymes remained unanswered because there was no report in roots either on redox proteins analogous to leaf Fd or concerning an electron transport system independent of the photochemical reactions. We identified two novel proteins in the roots: Fd-like electron carrier (REC = root electron carrier) and ferredoxin-NADP⁺ oxidoreductase (FNR)-like enzyme (PNR = pyridine nucleotide oxidoreductase). Without light energy in the roots, electrons are transferred, in the reverse flow compared to the situation around chloroplast PSI, from NADPH to PNR and then to REC for the reactions of NiR and GOGAT (Suzuki, Oaks, Jacquot, Vidal, & Gadal, 1985).
- As the environmental elements, e.g., light and nutrients are key signals to regulate inorganic nitrogen assimilation into amino acids, and plant growth and productivity (Beevers, 1981), we studied the light induction of Fd-GOGAT activity in greening etiolated leaves (Suzuki et al., 1982).

In these years in Orsay, the laboratory stepped into an advanced phase of study in the redox regulation and the molecular biology and genetics of the enzymes, and these pioneer studies were led by the groups of the carbon metabolism. Over this period, several lab mates worked week-ends as well to keep ongoing experiments as part of the overall effort. Pierre Gadal passed from time to time on Saturday and/or Sunday and exchanged with us on a variety of subjects or via a brief greeting when we were doing bench work. Sometimes, he left directly to the outdoor greenhouse behind Building 430 to relax himself through gardening, a favorite practice that he missed during his busy days. It is a contagious and distinctive trait of him to be able to share with the lab members his dedication to research by making them realize that what they undertake is indispensable. Such a sense of trust created a free atmosphere in the lab, as recalled by a former Master student of Nancy in her thesis preface: “I worked with my colleagues of the laboratory in a friendly atmosphere,” which sounds commonplace but is of high value (this was in one of the two master theses that Pierre Gadal handed to me in 1978).

3.1.3 In Versailles

The regulatory mechanisms in response to light and metabolites are major tools of the molecular and genetic studies in the analysis of nitrogen assimilation, biomass and plant productivity (Crawford & Arst Jr., 1993; Thomsen, Eriksson, Moller, & Schojoerring, 2014). The light and metabolite signals control the primary nitrogen metabolism throughout plant development in germinating seeds, vegetative organs, senescing and reproductive organs, and maturing seeds (Gaufichon, Reisdorf-Cren, Rothstein, Chardon, & Suzuki, 2010; Ireland & Lea, 1999). By molecular and genetic approaches, we dissected the regulation of isoenzymes of nitrogen assimilation located in the distinct cells, organelles and tissues by analyzing specific transgenic plants and mutants relative to:

- vegetative plants for the complex nitrogen flow from the NO₃⁻ reduction, photorespiratory NH₄⁺

- cycle and translocation of amino acids within the plant (see Review in Chapter 3) and reproductive plants for the synthesis of Gln, Glu, asparagine (Asn) and aspartate (Asp) and their remobilization within the plant.

We were able to show that light induces the specific mRNA transcription and protein synthesis of Fd-GOGAT in greening etiolated leaves: the study was carried out in a Canadian lab working with Pierre Gadal since the 1970s (Suzuki, Audet, & Oaks, 1987). It was later demonstrated that phytochromes mediate the induction of mRNA and protein of Fd-GOGAT via a reversible low fluence red/far-red treatment whereas NADH-GOGAT remained at a constant level (Suzuki et al., 2001; Ziegler et al., 2003). Using two different monoclonal antibodies against tobacco Fd-GOGAT, a competitive ELISA assay showed that the Fd- and MV-interacting domain share a common epitope (Suzuki, Vergnet, Morot-Gaudry, Zehnacker, & Grosclaude, 1994).

The first molecular study of GOGAT reported in 1987 concerned the cloning of a 6.3 kb NADPH-GOGAT gene of *E. coli* containing the 5'- and 3'-flanking regions and two ORFs for α large subunit (1514 amino acids of 166 kDa) and β small subunit (471 amino acids of 52 kDa) (Oliver et al., 1987). In the second half of the 1980s, increasing molecular and genetic studies were undertaken by different groups for enzymes of nitrogen assimilation in plants (Lam, Coschigano, Oliveira, Melo-Oliveira, & Coruzzi, 1996). Together with a Canadian group we succeeded at isolating a cDNA cloning for maize Fd-GOGAT (Suzuki, Burkhardt, & Rothstein, 1996) and then shifted to that of Arabidopsis as a model plant by working in this same lab in Canada. The cDNA sequence of Arabidopsis Fd-GOGAT was determined by screening cDNA clones isolated from a lambda ZAP II library and genomic clones from a lambda GEM-11 library, and the sequencing was carried out on both strands by the dideoxy-nucleotide chain termination method (Sanger, Nicklen, & Coulson, 1977). The complete cDNA of 5178 bp of *GLU1* gene, derived from a partial cDNA (4458 bp) complemented by the 5' sequence of a partial gene (1216 bp), encodes a single peptide of 1648 amino acid-protein (180 kDa) including the 131-amino acid transit peptide (14.6 kDa) (Suzuki & Rothstein, 1997). The structural comparison indicated that *GLU1* Fd-GOGAT may have originated from the bacterial α large subunit (Suzuki & Rothstein, 1997). *GLU1* Fd-GOGAT shares the N-terminal class II glutamine-aminotransferase (GAT) domain with *E. coli* NADPH-GOGAT (Oliver et al., 1987; Suzuki & Rothstein, 1997). Analysis of the Arabidopsis Genome Initiative (2000) indicated a 32 exon-intron structure of 9.57 Kb for the *GLU1* gene. Another Fd-GOGAT gene was suggested by our Southern blots of the restriction fragments of genome (Suzuki & Rothstein, 1997). Afterwards, the second *GLU2* gene of Arabidopsis Fd-GOGAT was characterized (Coschigano, Melo-Oliveira, Lim, & Coruzzi, 1998).

The cellular functions are tightly related to the cell-specific location. We dissected the spatial expression profile of Fd-GOGAT in the leaves by analyzing the Arabidopsis transgenic plants expressing a β -glucuronidase (*GUS*) reporter gene. Transgenic plants were generated by Gateway approach. The recombinant constructs of *GUS* fused with either *GLU1* promoter (2362 bp) or *GLU2* promoter (1580 bp) were electroporated into *Agrobacterium*, which were then inserted into the Arabidopsis genome by dipping floral tissues (Potel et al., 2009). *GUS* expression in mesophyll is consistent with the role of Fd-GOGAT in the primary NH_4^+ assimilation (Ireland & Lea, 1999) and photorespiratory NH_4^+ re-assimilation (Feraud et al., 2005; Somerville & Ogren, 1980). Moreover, *GUS* expression occurred in the phloem companion cell-sieve element (CC-SE) complex of minor leaf veins where signals of in situ mRNA hybridization visualized by a microscopy and the specific immunofluorescence by a confocal laser-scanning microscopy overlapped (Potel et al., 2009).

In the senescing and reproductive organs, the GS1/Fd-GOGAT cycle plays the primary role to maintain the Glu level in the phloem for nitrogen remobilization to the sinks (Masclaux-Daubresse et al., 2006). Four amino acids, Gln, Glu, Asn and Asp account for more than 50% of the total amino acids transported in the phloem (Gaufichon et al., 2013; Ireland & Lea, 1999; Valadier et al., 2008). Although the general view is that Asn is a long-distance nitrogen carrier, little is known about where Asn is synthesized, metabolized and translocated during the seed nitrogen filling. Asparagine synthase (AS) is encoded by a small multigene family in plants (Gaufichon et al., 2010), and *ASN1* encoding AS was more highly expressed in the reproductive organs of Arabidopsis than the other two genes (*ASN2* and *ASN3*) (Gaufichon et al., 2013).

Our molecular and genetic studies provided evidence for the roles of Asn in the reproductive organs during seed nitrogen filling. Quantitative changes of mRNA and metabolites were analyzed in the floral organ at three embryonic

stages from ovule (stage 0), globular (stage 1) and heart (stage 3) stages, and in the silique from heart (stage 4), torpedo (stage 7) and cotyledon and mature (stage 11) stages of *Arabidopsis* (Gaufichon et al., 2017).

- The profiling by HCA (hierarchical cluster analysis) and PCA (principal component analysis) was carried out by measuring mRNA by real-time RT-PCR for 16 selected genes of nitrogen and carbon metabolism such as *GLN2*, *GLU1*, *ASN1/ASN2/ASN3*, *ASPGA/ASPG* for cytosolic asparaginase (ASPG) releasing the Asn-amide group, and *AGT1* for peroxisomal asparagine aminotransferase (AsnAT) using the Asn-amino group (Gaufichon, Rothstein, & Suzuki, 2016). The onset of pollination caused a distinct mRNA clustering at stage 0 compared to the other stages due to the enhancement of mRNAs for *GLU1*, *ASN1*, *AspAT2* for aspartate aminotransferase (AspAT), *ASPGA1*, *ASPG1* and to a lesser extent of *AGT1* (Gaufichon et al., 2017).
- The profiling by HCA of 137 metabolites annotated by a gas chromatography-mass spectroscopy (GC-MS) differentiated stage 0 from the other stages according to sample maps using a distance linkage in the dendrogram and color density. This specificity was accompanied by the accumulation of Asn and the decline of Gln, Glu and Asp toward stage 1 (Gaufichon et al., 2017).

The spatial distribution of *ASN1* AS in the CC-SE complex detected by confocal microscopy in the silique cell wall was found to extend over the septum of the ovary along the central vascular strand, which branched to the ovule via the funiculus as well as the outer and inner seed integuments (Gaufichon et al., 2017). These results provided evidence that the post-phloem Asn synthesis in the CC-SE complex in the silique and septum meets a high demand for Asn for transit to its symplastic extension of seed integument during ovule differentiation into the embryo. Besides, the induction of *ASPGA1*, *ASPG1* and to a lesser extent *AGT* at the ovule stage implies an efficient flux of the amide and amino groups of Asn for Asp synthesis during the seed nitrogen filling. This Asn turnover is consistent with the phenotype of promoter *Napin2S::ASN1* lines expressing *ASN1* later up to the desiccating stage of seed maturation (*Napin 2S* for storage 2S albumin) (De Silva Conceição & Krebbers, 1994). The wild-type seed yields of the promoter *Napin2S::ASN1* lines (seed weight and seed number per silique) indicated that the promoter *Napin2S::ASN1* lines were impaired at the timing of Asn synthesis in the embryonic cells for flux of its amide and amino groups, while the *CaMV35S::ASN1* overexpressors had 5–9% higher seed yields (Gaufichon et al., 2017).

The other enzymes of NH_4^+ assimilation are co-localized in the companion cells including GS1, Fd-GOGAT, NADH-GOGAT, NADH-GDH and CPSase (Carvalho, Pereira, Sunkel, & Salema, 1992; Potel et al., 2009). As the companion cells in the photoheterotrophic reproductive organs contain a heterogenous mixture of photosynthetic chloroplasts and non-photosynthetic plastids at different sites of the same organ (DeWitt & Sussman, 1995), the amino acid synthesis in these cells requires an electron transfer system via NADPH/FNR/Fd in contrast to the chloroplast PSI driven as demonstrated in the roots (Suzuki & Knaff, 2005; Yoneyama & Suzuki, 2019).

An impact of Asn deficiency of the *ASN1* T-DNA mutant lines (*asn1*) was found in the defective differentiation of embryos from the images of whole-mount *asn1* embryonic cells, stained by pseudo-Schiff propidium iodide and analyzed by confocal microscopy according to Truemit et al. (2008). The aberrant embryo cell divisions in the upper suspensor cell layers (diagonal/additional divisions) and hypophysis surrounded by embryo proper and suspensor (transversal division) from globular to heart could be related to a deficiency of Asp as its entry into carbamoylphosphate is a limiting step in the uridine nucleotide synthesis (Gaufichon et al., 2017; Schröder, Giermann, & Zrenner, 2005).

Over this period, Pierre Gadad and I had many occasions to meet in Orsay, Versailles, Gif-sur-Yvette, Toulouse, Paris, Nara, Brittany, Nancy, etc. It was always a pleasure to witness his enthusiasm for research and to follow his comments and discussions. In the meantime, he accepted the demand of our scientific committee of France-Japan symposium organization, and coordinated the French delegation to the bilateral symposium at Nara, Japan (October 29–31, 2002). More than 100 attendees joined the meeting along with presentations of 18 and 16 leader scientists from France and Japan, respectively. He and his colleagues from longstanding collaborations confirmed that the two scientific communities share complementary research and methodologies and a common interest in certain model plants and crop species (CNRS-IRN France-Japan Frontiers in Plant Biology program 2020–2024). His advices to our scientific committee served for the subsequent symposia in Toulouse in 2005 (October 25–29, 2005) and Tsukuba, Japan in 2008 (October 19–24, 2008). He had already been in Tsukuba in the early 1980s and briefly shared a bit of springtime with my family on the way to Tsukuba to visit the newly opened research center on the campus. He paid attention to details

for lab members such as birthday celebrations, afternoon coffee breaks, numerous defense parties in the lab, etc. As far as I am concerned, his influence was determinant for the renewals of fellowship from the French government and short-term CNRS position, planning of postdoc stay, unwavering support for my application for an INRA position, elaboration of the symposium frames, etc. More recently, his dedication to research was still apparent as he agreed to be the Series Co-Editor of *Advances in Botanical Research* during 8 years (2012–2019) despite his long disease.

In September 2019, my wife and I went to visit him in the Cochin Hospital at Port-Royal. The day before, Mrs. Françoise Gadad confirmed that we could visit him. At the RER railway station, we found Mrs. Gadad in front of us. Her train from La Hacquinère should have coincided with the arrival of our train from Saint Michel-Notre Dame. She showed us the way to the Hospital that she took every day. In his room, he was checking the agricultural training report of his grandson. By staying with him and Mrs. Gadad for a while, we remembered about our walk with them on the Bayard street of Paris in the spring of 2017. They wished to visit the neighboring museum and its surroundings by extending the walk after lunch. We were very pleased to observe his large strides of walk, despite his battle against the disease, similar to some time ago at Foessant, Beg Meil and Ile Tudy in Finistère. Also, we called to mind a remembrance that he enjoyed a laborious pruning of the tall trees in his vast nursery close to the home in Brittany.

3.2 Bertrand Hirel's recollections

In 1974, the chloroplast enzyme Ferredoxin-dependent glutamate synthase (Fd-GOGAT; E.C. 1.4.7.1) was identified in plant tissues by Peter Lea and Benjamin Mifflin, two researchers based at Rothamsted Experimental Station in Harpenden, UK. This discovery drastically changed the existing ideas regarding the pathway of ammonium assimilation in plants (Lea & Mifflin, 1974). Before this discovery, it was generally considered that glutamate dehydrogenase (GDH, E.C. 1.4.1.3), an enzyme that had been known in plants since 1938 as reported by Smith, Austen, Blumenthal, and Nyc (1975), was chiefly responsible for the incorporation of nitrogen (N) into the α -amino group of amino acids using NADH as a cofactor (Bulen, 1956). In addition, Lea and Mifflin's research demonstrated that most of the ammonium that is metabolized by higher plants is assimilated via the glutamine synthetase/GOGAT (GS/GOGAT) cycle. This cycle carries out the incorporation of inorganic N into glutamate and glutamine, the two amino acids that are the fundamental molecules used for the synthesis of virtually all the compounds in plants that contain N. An important role for the enzyme GS was also proposed in the ammonia assimilatory pathway (E.C. 6.3.1.2), (Elliott, 1953). Later on, evidence for the key role of the GS enzyme demonstrated that irrespective of their origin, (nitrate reduction, dinitrogen fixation, photorespiration, protein and amino acid N recycling) ammonium ions are incorporated into the amide position of the amino acid glutamine in the presence of glutamate, via an ATP-dependent reaction catalyzed by the enzyme. This reaction, in conjunction with that catalyzed by GOGAT, is thus the major if not the only route facilitating the incorporation of inorganic N into organic molecules. GOGAT recycles glutamate and incorporates carbon skeletons into the GS/GOGAT cycle using the organic acid 2-oxoglutarate as a substrate (Hirel & Lea, 2001; Lea & Mifflin, 2018). In this coupled reaction, 2-oxoglutarate originates from photosynthetic CO₂ assimilation, via the glycolysis pathway and the Krebs cycle (Hodges, 2002)(Hodges, 2002), which represents a direct interface between C- and N-metabolism.

Following the discovery of the GS/GOGAT ammonium assimilatory pathway, it was thought that the GS enzyme was present as a single protein species located in the plastids, although enzyme activity was also found to occur in non-photosynthetic tissues such as roots (Kanamori & Mastumoto, 1972), thus indicating that there could be other forms of the enzyme, likely to be present in the cytosol (Mifflin & Lea, 1977).

Even following the discovery of the GS/GOGAT cycle, the possible role of mitochondrial NADH-dependent glutamate dehydrogenase in the process of ammonia assimilation was still put forward on a regular basis. This was due to the ability of glutamate dehydrogenase to fix ammonium in vitro utilizing the organic acid 2-oxoglutarate to synthesize glutamate. Some authors vehemently argued that GDH could be involved in the assimilation of inorganic N in plants, even though its K_m for ammonium was at least 10 times higher than that of GS (Oaks, 1995). It was also proposed that under certain physiological conditions to avoid a build-up of ammonium ions, the enzyme is able to synthesize glutamate (Skopelitis et al., 2006). However, so far, all the experiments performed *in planta*, using 15N-labeled techniques, have demonstrated that GDH operates in the direction of glutamate deamination (Stewart, Shatilov, Turnbull, Robinson, & Goodall, 1995), even when the enzyme is overexpressed several times in transgenic plants (Labboun et al., 2009). It was therefore concluded that GDH is involved in the recycling of carbon molecules by supplying 2-oxoglutarate to tissues that are carbon limited, rather than in the assimilation of ammonium when carbon is

in excess, a finding which was largely confirmed using Arabidopsis mutants totally devoid of GDH activity (Fontaine et al., 2012).

I joined Pierre Gadal's group as a Masters student at the University of Nancy, France, in 1979. Following the Rothamsted group in the UK, Pierre Gadal set up one of the pioneer teams working on plant N-metabolism in France. The idea behind the development of such research was to improve N use efficiency (NUE) in plants both for **Q19** agronomic and ecological purposes (Hirel & Krapp, 2020)(Hirel and Krapp, 2019).

Pierre Gadal first asked me to demonstrate that non-symbiotic N₂-fixing bacteria such as *Azospirillum* could provide N to plants, in a similar manner that can occur in legumes. Some of these bacteria were isolated by a group located nearby at the Centre de Pédologie Biologique of the CNRS (Bally, Thomas-Bauzon, Balandreau, Richard, & De Rey, 1983). As my investigations were not really conclusive, this diminished my motivation to pursue further research on the topic. Pierre Gadal then asked me to move my research to the biochemical characterization of GS. Later on, other members of Pierre Gadal's group, using a sophisticated gnotobiotic system, managed to show that part of the bacterial N originating from atmospheric N₂ acted as N fertilizers, thus allowing the demonstration of a complete plant developmental cycle (Maudinas, Chemardin, Yovanovitch, & Gadal, 1981). Interestingly, a simplified version of this gnotobiotic system is currently used by my group to study the interaction between non-symbiotic N₂-fixing bacteria and maize (Brusamarello-Santos et al., 2017). Ironically, this topic which did not excite me so much at the early stage of my career, has become one of the most extensively studied fields of plant research, principally due to the promising applications in sustainable agriculture to limit the overuse of N fertilizers that are detrimental to the environment (Dellagi, Quilleré, & Hirel, 2020).

Following the demonstration that in higher plants, ammonia is incorporated into organic molecules via the GS/GOGAT pathway, two groups discovered more or less simultaneously, the occurrence of two types of glutamine synthetase isoenzymes, one located in the cytosol and the other in the plastids. The two GS isoenzymes were originally identified by two PhD students (Caroline Guiz in Pierre Gadal's group and Fred Mann in George Stewart's group) using ion exchange chromatography experiments in which two peaks of enzyme activity could be separated in rice and barley **Q20** leaves (Guiz, Hirel, Schedlofsky, & Gadal, 1979; (Mann, Fentem, & Stewart, 1979) Mann et al., 1979). The first one eluted from the column was thus called GS1 and the second one GS2. Detailed biochemical analyses, the kinetic properties of the two peaks of enzyme activity (Hirel & Gadal, 1980a) and identification of their subcellular localization revealed that GS1 and GS2 were different proteins, the first one located in the cytosol and the second one in the **Q21** plastids (Hirel & Gadal, 1980b; (Wallsgrave, Lea, & Miflin, 1979) Wallsgrave et al., 1979). Spinach leaves were first used to perform cellular fractionation in order to locate the two GS isoenzymes, because it was relatively easy to isolate intact chloroplasts in this plant using gradients of aqueous solutions containing increasing concentration of sucrose. However, following ion exchange chromatography, only one peak of GS activity could be detected in this species, which started to worry us about the real occurrence of two isoenzymes of the GS enzyme in plants. However, more difficult to handle, non-aqueous fractionation techniques were successfully used to demonstrate that GS1 was located in the cytosol while GS2 was present in the plastids (Hirel & Gadal, 1980a), a result confirmed later in pea which also contained the two forms of the enzyme in leaves (Hirel & Gadal, 1981).

The puzzling differences observed between spinach and rice with respect to their GS isoenzyme content, prompted me to examine other plant species including C₄ plants such as sorghum. Sorghum was at that time one of the favorite model species used to study the enzyme phosphoenolpyruvate carboxylase by Pierre Gadal's team (Vidal, Cavalie, & Gadal, 1976). This led Pierre Gadal to make the comment, "this is horizontal research, you should concentrate on the characterization and on deciphering the physiological role of GS1 and GS2." However, we soon realized that looking at different plant species was the right thing to do first, as we found that in sorghum the relative proportions of GS1 and GS2 were similar (Hirel & Gadal, 1982). This led to the conclusion that the GS isoenzyme complement was species-specific and to the hypothesis that their physiological role was also different, depending on whether they were C₃ or C₄ plants.

In 1981 at a meeting of the Society for Experimental Biology in Leicester, our group (from the University of Paris-Orsay, France) and that of George Stewart (from Birkbeck College, University of London, UK) recognized the similarity of their studies, and decided to publish a joint paper (McNally, Hirel, Gadal, Mann, & Stewart, 1983). This also marked the beginning of the most productive period of research I can remember, leading to the publication of seven joint papers from 1983 to 1984 which also led to the development of many close friendships. Certainly, the

subsequent collaborative work that we pursued proved no less rewarding-scientifically, gastronomically, and enologically.

Together with the discovery of GOGAT, this new finding strongly rekindled the interest of plant biologists to reconsider the importance of inorganic N assimilation in plants and its regulation considering both the tissue and subcellular localization of the different GS isoenzymes. This also transformed glutamine synthetase from a rather mundane enzyme, previously considered as being mostly involved in the synthesis of the storage compound glutamine, into a key enzyme of plant N-metabolism. We were able to show that plant species differed with respect to the occurrence and relative amounts of glutamine synthetase isoenzymes. We found that four groups of plants could be recognized on the basis of differences in the stoichiometry of the isoenzymes in leaf tissue. One of the most important findings was that many plants with C₃ photosynthesis lacked the cytosolic isoenzyme of glutamine synthetase. In contrast, most of the C₄ species that we examined had a high content of the cytosolic isoenzyme. The importance of this lay in the proposal from the Rothamsted group that photorespiratory ammonium was reassimilated via the cytosolic isoenzyme (Keys et al., 1978). Our results ruled this out for many species, leaving a role in the photorespiratory N cycle for chloroplastic glutamine synthetase because of the lack of GS1 activity in the cytosol of leaf parenchyma cells in a number of C₃ plant species (McNally et al., 1983). This finding was further confirmed by immunocytochemical studies using antibodies raised against the GS protein that recognized both GS1 and GS2 (Hirel, Perrot-Rechenmann, Suzuki, Vidal, & Gadal, 1982). Ironically, the most decisive evidence confirming our hypothesis came from mutants of barley isolated by both the Rothamsted and Lancaster groups that lacked chloroplastic glutamine synthetase and could only grow under conditions that suppressed photorespiration (Blackwell, Murray, & Lea, 1987; Wallsgrove, Turner, Hall, Kendall, & Bright, 1987).

Later on, a research group from Portugal using immunogold transmission electron microscopy was able to show that a small proportion of GS1 was present in the cytosol of the phloem companion cells (Carvalho et al., 1992), a finding which turned out to be a general feature in all higher plants examined so far. The involvement of GS1 in the phloem in the control of long-distance transport of N-containing molecules such as proline was further deciphered by a number of groups for example using transgenic plants impaired in cytosolic GS activity in this tissue (Brugière et al., 1999; Caputo, Victoria Criado, Roberts, Geslo, & Barneix, 2009; Kamachi, Yamaya, Hayakawa, Mae, & Ojima, 1992).

Q22 The original work by McNally et al. (McNally et al., 1983) (1981) has been frequently cited over the last 3 decades because it provides a comprehensive account of the occurrence of glutamine synthetase isoenzymes in plants having different physiological and ecological characteristics in terms of plant adaptation to a particular habitat, to various environmental conditions, and in different tissues during the plant developmental cycle from germination to senescence (Cren & Hirel, 1999).

Later on, the main reason for the success concerning the discovery of the GS isoenzymes was the rise of plant molecular biology. In the two decades since the original biochemical findings of McNally et al. (1983), an increasing number of studies carried out on different legume and non-legume species have been developed at the molecular level by a number of groups including ours own. The aim of these studies was to further decipher the role of the different GS isoenzymes and the regulation of their synthesis and the assembly of their different subunits during N assimilation and

Q23 N remobilization (Masclaux, Quilleré, Gallais, & Hirel, 2001; (Yamaya & Kusano, 2014) Yamaya and Kusano 2014). One of the main milestones in these studies was the cloning of a cDNA encoding the cytosolic form of the enzyme present in large amounts in nodules of legume plants. Such cloning was facilitated by the fact that the transcripts encoding GS1 were strongly induced in N₂-fixing nodules. This is due to the large amount of ammonium produced by the enzyme nitrogenase that is first incorporated into glutamine to allow the synthesis of N-containing molecules in the

Q24 form of ureides that are further exported to other parts of the plant (Cullimore et al., 1984). Homologies of sequences between the different cDNAs encoding GS1 and GS2 then allowed the cloning of the corresponding transcripts from different plant species (Cren & Hirel, 1999).

Other remarkable pieces of work at the molecular level were the use of a reporter gene to show the tissue-specific synthesis of the GS isoenzymes in organs such as nodules, leaves or roots of transgenic plants (Miao, Hirel, Marsolier, Ridge, & Verma, 1991). Strong evidence for the putative role of GS in plant productivity was first demonstrated following one of the pioneer studies based on the exploitation of maize genetic diversity and the detection of QTLs. Rather unexpectedly, QTLs for grain yield were found to colocalize with QTLs for leaf GS activity and structural genes encoding the cytosolic form of the enzyme (Hirel et al., 2001). This led to the proposal that GS1 could be a

major driver involved in the control of grain yield, which prompted the development of numerous genetic manipulations in a variety of plant species to increase the activity of the enzyme in order to boost plant performance. As such, GS became one of the most intensively studied enzymes in terms of NUE enhancement both in monocot and dicot plants. In species such as rice, maize, barley, pea and poplar, transgenic plants overexpressing GS1 or GS2 displayed improvement in NUE, increased biomass and higher grain yield (James, Borphukan, Fartyal, Achary, & Reddy, 2018; Plett, Garnett, & Okamoto, 2017). One of the most remarkable results was the increase in grain yield obtained in maize plants overexpressing GS1 and a reduction in yield observed in mutants deficient for two forms of the cytosolic enzyme (Martin et al., 2006). This study was another example of another remarkable collaboration established between my group and two other groups based in the UK, namely those of Keith Edwards at the University of Bristol and Peter J. Lea at Lancaster University. As a conclusion, my personal feeling about the pioneer work conducted when I was in Pierre Gadad's laboratory on the process of ammonia assimilation involving the discovery of the different GS isoenzymes, is that it illustrates how rewarding collaborative science can be. Such a collaboration has involved the synthesis of the findings of several colleagues in the UK and has proved far more scientifically significant than if the research had been published independently.

Acknowledgements: We are particularly grateful to Professor Peter J. Lea, who was one of the pioneer scientists in the field of nitrogen assimilation as well as an outstanding collaborator for more than 20 years, for critically reviewing the manuscript.

4 Carbon-nitrogen interactions and isocitrate dehydrogenases

4.1 Ridong Chen's recollections

NADP-dependent isocitrate dehydrogenase and α -ketoglutarate needed for nitrogen assimilation in plants. After having graduated with a bachelor from a Chinese University in the summer of 1984, I was chosen out of many talented students to gain the privilege for studying abroad. In the spring of the following year, I traveled to France, carrying with me poor French but a big dream. That dream was to convert the infinite resource of sunshine into an abundance of electrical energy. I was initially accepted in the Master program of biophysics at the Center of Atomic Energy. However, an insufficient education in advanced mathematics and quantum mechanics compelled me to switch to the Master program in plant molecular biology and biotechnology, led by Pierre and Francis Quétier. I met Mr. Gadad in his office after arriving in Paris from Language School of Montpellier. In my first impression, he was a tall, energetic gentleman. He told me he had checked my background with full satisfaction by writing to my Chinese professor of plant biochemistry. After spending several dull months applying 2D gel electrophoresis to study proteomic changes during light-induced greening of plants with Jean Vidal and an INRA team, I wanted to change direction and pursued research on restriction enzymes for my doctoral degree. In the summer of 1986, Pierre invited me several times to discuss about my thesis work in his lab. I showed no interest, as at the time, protein research was no longer considered advanced. In other words, it was unfashionable. With his persistence, and his recruitment of one friend to convince me, we finally met in his office. My eyes glazed over as the conversation quickly turned to isocitrate dehydrogenase, purification, and enzymes. Bluntly, I asked him why I should work on this. He told me that this enzyme might be the key to improving nitrogen assimilation and crop yield. My interest piqued, and as he described to me the possibilities, my imagination began to run wild with images of important discovery, perhaps even a new metabolic pathway in a textbook.

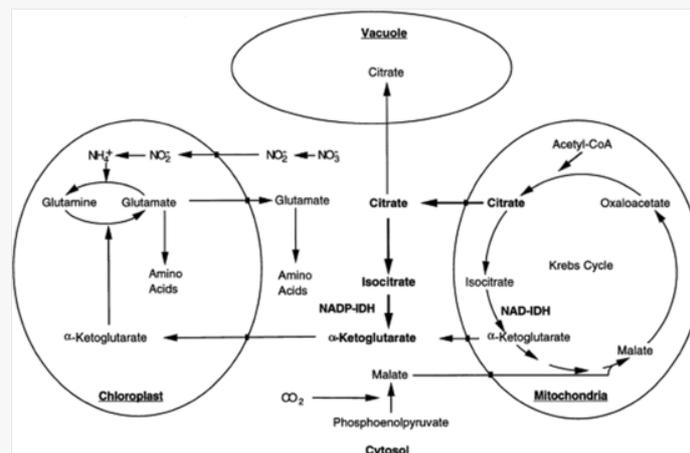
In the late summer of 1986, I had naught on my mind but the purification of the protein. After 3 months of hard work, I managed to purify small quantity of protein to homogeneity, made possible by the guidance of Pierre and the help of certain publications. I was so excited that I enthusiastically invited my graduate student friends to view the “beauty” of a faint blue band on the gel. I then spent 6 months working day and night in the cold room of the basement, often staying until the wee hours of the morning, monitoring chromatographic processes. With the precious 200 μ g protein purified from tons of fresh pea leaves, Jean Vidal helped me immunize three rabbits, with one producing good titer of polyclonal antibodies. I compared the kinetic and immunological properties of the enzymes purified from roots and leaves. Pierre and I worked with great joy and together published our very first paper in *European Journal of Biochemistry* in 1988 (1). With the help from a kind and competent technician named Evelyne Bismuth and chromatographic and immunological tools, we discovered that a distinct isozyme was present in the isolated chloroplast (2). Evelyne and I managed to purify a sufficient quantity of the chloroplast isozyme for analysis and a manuscript was ready for submission. However, due to the presence of a band of 80 kDa on the gel, we could not be certain whether

chloroplastic isozyme was a monomer of 80 kDa or a dimer of 2×40 kDa. For the sake of avoiding the possibility of reporting false findings and for peace of mind, the manuscript was never published. With the help from Evelyne and two motivated undergraduate students, Emmanuelle and Claire, I went out to the forest near Orsay, collected plant leaves, and then identified species with the generous help from a Professor of the fourth floor. We showed that NADP-isocitrate dehydrogenase was present and immunologically related in all the lower and higher plants we surveyed (3). I also initiated determination of the ratio of chloroplastic vs cytosolic isozymes and eventually completed the work when I became a professor of Biochemistry in Canada (4). With the help of Claude Crétin, I isolated a partial cDNA and concluded the experimental work of my 3-year doctoral research.

At the time I started to write up my thesis, an aconitase isozyme was also reported in the plant cytosol. This, along with the presence of citrate in the cytosol, citrate transporter on the mitochondrial membrane, and lack of α -ketoglutarate-degrading enzyme in cytosol, inspired me to propose a novel pathway in the cytosol. With Pierre's encouragement and enthusiastic participation, we published the new concept of "division of labor": NADP-isocitrate dehydrogenase and the cytosolic pathway provide NADPH and α -ketoglutarate for biosynthesis, while NAD-isocitrate dehydrogenase and Krebs cycle produces NADH and α -ketoglutarate for ATP generation (5, and see Fig. 4). Pierre and I also co-authored a review paper, the very first systemic review of this enzyme in plants and other species (6). Seven years later, our cytosolic pathway was confirmed with NMR by a German team and adopted by the textbook of *Plant Biochemistry and Molecular Biology* (7). The dream had come true!

alt-text: Fig. 4

Fig. 4



Q1

Critical role of cytosolic NADP-dependent isocitrate dehydrogenase in providing α -ketoglutarate needed for plant nitrogen assimilation.

Pierre was a kind, joyful, and sometimes humorous man. Over the 3 years of interaction, we occasionally talked about personal life. I remember 1 day as he shared about how he had met his wife. He had spotted her in her father's dentist office, and immediately fell in love. He was very proud of being granted the title of professor at the age of 31, a rare achievement in the French education system. I feel a sense of warmth, as I think back on the good times we shared as friends during my time at Orsay. When Pierre left for sabbatical leave at the Washington University, I felt lonely as it was often difficult to get in touch with him. I was also mildly frustrated, since sometimes I was unable to order research reagents without his approval. Nevertheless, it worked out in the end, as this forced me to read more which was very

helpful for the review article later on. I remember that every time I asked him when he would retire. He said loudly “Chen, I am only fifty something.”

After I left the lab, Pierre remained a good friend and provided unconditional support for my faculty applications in the USA and Canada. We exchanged greetings and family pictures during Christmas time. I visited him in 2002 when I had business in France. He came to pick my family up from the train station and proudly showed me the new Plant Biotech Institute he and Francis established. My wife and I were fortunate enough to see him again in his house during the Summer of 2018 and I will remember fondly our 90 min conversation in rusty French. Despite fighting cancer for some time and recent surgery, he remained joyful and optimistic. He was very curious about what I had been doing these years. I shared with him how much I was blessed and how much I enjoyed my biomedical discovery. He emphasized that there was still so much to do for plant scientists in order to secure food safety for humanity. We left with repeated promise of “seeing each other again” while he was standing at the entrance of the house.

A few months before he passed away, he informed me of his new email account and comforted me by saying “he is doing great.” In spite of challenges of both language and culture, I consider my Orsay journey with Pierre as both unexpected and amazing. This allowed me to enter the fascinating enzyme world and find something to build on. I will dearly miss and warmly remember Pierre as a great mentor and a very good friend.

4.2 Michael Hodges's recollections

In 1988, I, Michael Hodges that is, was working at the CNRS campus in Gif-sur-Yvette and already collaborating occasionally with Myroslawa Miginac-Maslow when my laboratory was closed for renovation as part of the creation of a new Institute to be called the ISV (Institut des sciences du végétal). It was under these circumstances that Pierre Gadad allowed me to join his laboratory at the Université Paris-Sud in Orsay to work on the purification of LHC kinase with Myroslawa. I then went on to investigate FNR (ferredoxin-NADP reductase) phosphorylation before deciding to move on to thioredoxins with Jean-Pierre Jacquot where I notably worked on a thioredoxin f from pea (Hodges et al., 1994) using a cDNA isolated by Loïc Lepiniec when he was screening for PEPC! I was a biophysicist by trade and joining the laboratory of Pierre Gadad gave me the opportunity to discover and to learn some biochemistry and protein purification techniques. This was then further extended to molecular biology, in the days when the laboratory had a couple of classic MacIntosh computers with Tetris inside and PCR was done using water baths before the first PCR machine was bought!

I ended up working at a laboratory bench in a room at the end of the corridor where I found myself sharing bench space with Susana Galvez Garcia, a Spanish student who had come from the laboratory of Francisco Canovas in Malaga to do her PhD with Pierre. She was given the task of characterizing NADP-dependent isocitrate dehydrogenases (ICDHs) after R-D. Chen had successfully finished his PhD and left. This enzyme had become a favorite of Pierre Gadad because it produced 2-oxoglutarate (2OG) and he assumed it to be the missing link between carbon metabolism and nitrogen metabolism since ammonium assimilation by the GS/GOGAT cycle required this keto-acid to make glutamate. At the time, Pierre Gadad was preoccupied with creating the IBP thus leaving Susana just a little bit under-supervised and so I decided to help her out with discussions and advice. Who would have thought that this was to be the start of a long story leading to us getting married and sharing more than just memories about a common research goal?

Before my involvement, Susana had already purified and characterized cytosolic and chloroplastic ICDH isoforms from mixotrophic tobacco cell suspensions (Galvez, Bismuth, Sarda, & Gadad, 1994). The idea of using this biological material was the brain child of Evelyne Bismuth, who had worked with Ridong Chen and now she was giving Susana some technical support. It was then decided by Pierre Gadad that Susana should develop a more modern molecular biology strategy. The first aims were to identify and isolate ICDH cDNA clones. Having no experience, Susana was lucky to be taken under the wings of Claude Créatin who helped her to make a cDNA library using mRNA isolated from tobacco cell suspensions. Claude became a dear friend but this was cut short by his untimely death. It was around this time that Susana moved from the basement to the laboratory bench in the room at the end of the corridor on the first floor of the building number 430 that I was using. First, Susana screened her cDNA library using antibodies raised against her purified ICDH proteins from cell suspensions but this strategy was unsuccessful. Eventually, several ICDH cDNA clones were isolated using a probe made from a partial soybean ICDH cDNA generously sent by Dr. M. Khan of Washington State University. Full-length cDNAs were then generated using PCR fragments amplified from the cDNA library. Based on the sequencing of tryptic peptides from purified tobacco ICDH isoforms a correspondence

was made between cytosolic ICDH and an isolated cDNA (Gálvez et al., 1996). This allowed the production, purification and characterization of recombinant tobacco cytosolic ICDH (Gálvez et al., 1995). Although, my failing memory does not allow me to say what my actual contributions to this work were, I ended up as co-author of both papers cited above. During this period, Susana had met Sabine Fieuw at a meeting where she learnt that Sabine was working on ICDH in the group of Lothar Willmitzer at the MPIMP Gölm Germany. Together, they isolated a cytosolic ICDH cDNA from potato using our tobacco iCDH cDNA as a probe (Fieuw, Müller-Röber, Gálvez, & Willmitzer, 1995).

During our time in the building number 430 at the Université Paris-Sud in Orsay, we were also involved with some of Pierre Gadals friends: Francis Martin at the University of Nancy sent Vincent Boiffin to the laboratory to clone ICDH from Eucalyptus (Boiffin et al., 1998) and Rajbir Sangwan at the University of Picardie in Amiens helped us to immuno-localize ICDH by electron microscopy (Gálvez et al., 1996). Rajbir remained a friend over the years with several invitations to join PhD juries and recruitment committees as well as to correct the English of his numerous publications.

Anyway, back to my story. We could be proud of our ICDH discoveries and the first part of my ICDH story ended in 1994 when Susana obtained her PhD in the new amphitheater of the IBP that Pierre Gadad had convinced influential people to build. I can remember that I made some Indian food for the laboratory celebration and the day finished with an enjoyable typically French meal with Pierre, Susana, and Francisco Canovas at a nearby restaurant at Villiers-le-Bâcle that Pierre Gadad had chosen. It was the last PhD supervised by Pierre Gadad and after it was defended, Susana stayed in the team as a post doc paid for by an EU grant obtained by Pierre Gadad where we found ourselves in a consortium composed mainly of people working on fructans. This money allowed us to carry on working on ICDHs, to visit Copenhagen for a consortium meeting and to help organize the following one at the IBP, and to learn about fructans. During this period, a major aim was to show that one of our isolated tobacco ICDH cDNAs encoded the chloroplastic isoform. This involved developing in vitro translation and import of the resulting ICDH protein into isolated chloroplasts and eventually transforming tobacco to express an ICDH-GFP fusion protein. Together, these experiments strongly suggested that our cDNA encoded a mitochondrial ICDH and not the chloroplastic isoform but at the end of the day we were over the moon because our work was published in the prestigious journal, *Proc. Natl. Acad. Sci. U.S.A.* (Gálvez et al., 1998). That said, it is now admitted that the ICDH gene we were working on actually encodes an ICDH co-localized to both chloroplasts and mitochondria. Some unpublished work by Valérie Flesch at the IBP indicated that this was probably due to two translation start sites that defined the specificity of the transit peptide involved in addressing the preprotein to the organelles. We also adventured into making transgenic tobacco plants with highly reduced cytosolic ICDH activity but they did not show any growth phenotype! This made us think that perhaps it was mitochondrial NAD-dependent isocitrate dehydrogenase (IDH) that was producing the 2OG required for ammonium assimilation. This became the focus of the thesis of Muriel Lancien, and we were the first to identify IDH cDNAs and to show by the complementation of yeast *idh* mutants that tobacco IDH was heteromeric and required at least two different subunits to be active (Lancien, Gadad, & Hodges, 1998). In collaboration with Bertrand Hirel, a former member of the Gadad laboratory, who was working at the “Institut Jean-Pierre Bourgin” (IJPB) in Versailles, we investigated the nitrate-response of IDH genes (Lancien et al., 1999).

At the end of Muriel's PhD thesis, I was given the opportunity to manage my own research team at the IBP. After a 4-year break to investigate mitochondrial organic acid transporters, work on IDH continued during the thesis of Thomas Lemaitre and by this time it had been decided to move away from tobacco and to use Arabidopsis to benefit from mutant libraries and the genome sequence that had indicated the presence of five functional IDH genes. Again, single IDH mutants did not show any growth phenotype under standard laboratory growth conditions (Lemaitre et al., 2007). Although the isocitrate dehydrogenase topic at Orsay was initiated by Pierre Gadad, the Arabidopsis IDH work was no longer associated with him. However, we still interacted up to and beyond his retirement with friendly discussions in the corridors of the IBP, and every time Pierre crossed my path he would enquire about the latest news concerning me and Susana. I am most grateful to Pierre Gadad because he welcomed me into his laboratory, he gave me the liberty to decide which research areas to pursue and finally he allowed me to take over his original ICDH story and to go on to develop my personal research interests. This started at the end of the 1990s and the identification and characterization of mitochondrial organic acid transporters with Nathalie Picault, a Master then PhD student, and our Italian collaborators, Fernando and Luigi Palmieri (Picault, Palmieri, Pisano, & Palmieri, 2002) (Picault et al., 2002). It continued with further studies on 2OG but this time as a possible signal modulating N-metabolism via the PII protein.

This work was done in collaboration with Sylvie Ferrario-Méry and Sebastien Baud of the IJPB in Versailles and by Ana-Belén Feria Bourrellier, a Spanish Post doc whom Jean Vidal knew. The PII story and my love for 2OG ended with me spending 10 months in hospital and two major discoveries: arginine and fatty acid biosynthesis are regulated by a 2OG-dependent interaction of PII with *N*-acetyl glutamate and acetyl-CoA carboxylase, respectively (Feria Bourrellier et al., 2010; Feria Bourrellier, Ferrario-Méry, Vidal, & Hodges, 2009). If it was not for Pierre Gadal, I would have never become acquainted with this keto-acid and I would have never pursued so many happy years of my research career trying to elucidate how it was made, how it was transported and how it could be a signal to regulate plant metabolism.

5 Concluding remarks

Shortly after this series of recollections was initiated many of the former students and colleagues of Pierre Gadal have expressed interest in participating. All of them could not do it for a variety of reasons but there is no doubt that their recollections would have expressed the same feeling. First, we thought that the best way to honor Pierre was to report the science that has been done in his lab as he was foremost a researcher. Hopefully the readers will get more than a glimpse of how it's been done, why it's been done that way and of the connections between the many researchers involved. In many ways Pierre has altered our scientific paths but also more broadly our lives. The readers will have noticed similar terms coming all over again, benevolence, simplicity, tenacity and unwaivering support for his students. Following the example given by Pierre large freedom of expression has been given to all contributors and the resulting article is inherently rather heterogenous. Still all of us have written it with their soul and guts and in the end, we hope that we have paid to Pierre the tribute that was so well deserved.

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 The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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Q29 Further reading

 The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

Chen, R.D. (1998). Plant NADP-dependent isocitrate dehydrogenases are predominantly localized in the cytosol. *Planta*, 207, 280–285.

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Keywords: Fructose biphosphatase; Glutamine synthetase; GOGAT; Isocitrate dehydrogenase; NADP-malate dehydrogenase; Nitrogen assimilation; PEP carboxylase; Photosynthesis; Redox regulation; Thioredoxin

Queries and Answers

Q1

Query: Figure 04 is not in printable quality. We suggest to redraw at our end to improve quality. Please confirm to proceed with our suggestion else provide better quality figure.

Answer: Please find a better quality image for Fig 4

Q2

Query: Please check whether the suggested running head "Scientific contributions of Pierre Gadal and his lab" is appropriate for this chapter.

Answer: This is correct

Q3

Query: Please note that "Frederic" has been treated as initial and "De Lamotte" has been treated as surname in the author name "Frederic De Lamotte". Please check and confirm if it is correct.

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Q4

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Answer: Correct

Q5

Query: Please check that we have captured all author names and affiliation details correctly.

Answer:

Q6

Query: Please provide a affiliation details for the author "Tatsuo Sugiyama".

Answer:

Tatsuo Sugiyama is now retired from Nagoya University. The only supplementary information I could provide is his private address : cedertree@nifty.com

Q7

Query: Please check whether the hierarchy of the section levels is appropriate as given in this chapter.

Answer: Correct

Q8

Query: Ref. "Miranda-Vizuet et al., 1997" is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Done

Q9

Query: The citation "Jacquot et al., 1997" has been changed to "Jacquot, Lopez-Jaramillo, Miginiac-Maslow, Lemaire, Cherfils, Chueca, et al., 1997" to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.

Answer: Correct

Q10

Query: The citation “Mittard et al., 1994” has been changed to “Mittard et al., 1995” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.

Answer: Correct

Q11

Query: Ref. “Garcia-Sanchez et al., 2000” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Citation added in the list according to : García-Sánchez, M.I., Gotor, C., Jacquot, JP. *et al.* Glutamate synthase from *Chlamydomonas reinhardtii*: Interaction studies with its substrate ferredoxin and molecular cloning. *Plant and Soil* **221**, 59–65 (2000). <https://doi.org/10.1023/A:1004715504065>

Q12

Query: The citation “Lemaire et al., 1999” has been changed to “Lemaire, Hours, et al., 1999; Lemaire, Keryer, et al., 1999” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.

Answer: Correct

Q13

Query: The citation “Chen et al., 1989” has been changed to “Chen, Bismuth, Champigny, & Gadal, 1989; Chen, Bismuth, Issakidis, et al., 1989” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.

Answer: Correct

Q14

Query: Please provide chapter title followed by author names in place of “Chapter 4”.

Answer: Chapter 4: Glutamate synthase and amino acid synthesis in higher plants. Author: Akira Suzuk

Q15

Query: The citation “Hoagland (1938)” has been changed to “Hoagland and Arnon (1938)” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.

Answer: Correct

Q16

Query: The citation “Lowry, 1951” has been changed to “Lowry, Rosenbrowgh, Farr, & Randall, 1951” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.

Answer: Correct

Q17

Query: Please provide chapter title followed by author names in place of “Chapter 3”.

Answer: Chapter 3: Scientific contributions of Pierre Gadal and his Lab. Nitrogen metabolism pathways and regulation - Akira Suzuki’s recollections. Author: Akira Suzuki

Q18

Query: Ref. “Hodges, 2002” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Citation added in the list and inserted in text

Q19

Query: Ref. “Hirel and Krapp, 2019” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Date corrected (2020 instead of 209) and reference added in the list and in text

Q20

Query: Ref. “Mann et al. 1979” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Done

Q21

Query: Ref. “Wallsgrave et al., 1979” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Done

Q22

Query: Ref. “McNally et al. (1981)” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

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Q23

Query: Ref. “Yamaya and Kusano 2014” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: Done

Q24

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Answer: Correct

Q25

Query: Ref. “Picault et al., 2002” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text.

Answer: I added the reference in the list according to this :

Picault N, Palmieri L, Pisano I, Hodges M, Palmieri F. Identification of a novel transporter for dicarboxylates and tricarboxylates in plant mitochondria. Bacterial expression, reconstitution, functional characterization, and tissue distribution. J Biol Chem. 2002 Jul 5;277(27):24204-11.

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Epub 2002 Apr 26. PMID: 11978797.

Q26

Query: Please check whether the inserted volume and page numbers are appropriate in the reference “Quilleré and Hirel (2020)”.

Answer: Correct

Q27

Query: Please provide publisher name for the reference “Lockard (1959)”.

Answer: Done

Q28

Query: Please check whether the edit made to the volume number and the inserted page number are appropriate in the reference “Ziegler et al. (2003)”.

Answer: Correct

Q29

Query: Further reading section contains references that are not cited in manuscript text. Please cite the references or confirm if we could delete it? Further reading will be retained if the references are not cited or deleted.

Answer: